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THE FORMATION OF PANCREATIC JUICE PROTEINS STUDIED WITH LABELLED AMINO ACIDS

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Introduction

The exocrine part of the pancreas is adapted for the synthesis and secretion of digestive enzymes. The mechanism for the secretion of pancreatic juice has been the subject of extensive studies. PAVLOV and his associates (PAVLOV 1898) demonstrated the significance of the nervous mechanism, and the regulation of the secretion was clarified in some degree by the discovery of secretin (BAYLISS and STARLING 1902) and pancreozymin (HARPER and RAPER 1943). Our knowledge is still very imperfect, however, concerning the mechanism for discharge of the secretion and synthesis of the enzymes.

Microscopic studies have long since shown that cells of the secretory glands, including the exocrine cells of the pancreas, contain characteristic granules (HEIDENHAIN 1875), and that these granules decrease when the pancreatic secretion is stimulated and the digestive enzymes are discharged into the intestines. Although the resynthesis of zymogen granules has been widely investigated, both with the use of histologic preparations (HEIDENHAIN 1875, and others) and by microscopic examinations in living animals (COWELL 1928, and others), we still know little about that process.

During the last decade investigations with labelled amino acids have shown that the bulk of the proteins are synthesized by free amino acids (LOFTFIELD 1957, and others); the latest studies indicate that most, and probably all, of the pancreatic juice proteins are enzyme proteins (KELLER, COHEN and NEURATH 1958). The writer, in a preliminary report (HANSSON 1957), found that the concentration of radioactivity was higher in the pancreas than in other organs following injection of S^{35}-methionine and that, when the latter was injected intravenously, the radioactivity was incorporated into the pancreatic juice proteins.

It seemed possible, judging by these results, to study the synthesis of pancreatic juice proteins by using labelled amino acids as precursors of the digestive enzyme proteins. The present investigations were mainly designed to elucidate the rate at which the digestive enzymes are synthesized. Labelled amino acids alone were employed as precursors for studying the formation of the enzyme proteins.—Methionine has been used in most investigations because it can be labelled with the radioactive isotope S^{35}, whose soft beta radiation makes it especially suited for autoradiography. Methionine occurs in all enzyme proteins of pancreatic juice that have so far been studied (NORTHROP, KUNITZ and HERRIOTT 1948; GREEN and NEURATH 1954; CALDWELL *et al.* 1954).

The pancreatic uptake and distribution of labelled amino acids and their incorporation into the proteins of various cell fractions can be followed. It may also be possible to localize the formation of the labelled proteins in the cell, and movements of radioactivity therein can be determined by observing the course at intervals. Moreover, the excretion of proteins formed in the pancreas can be studied by following the radioactivity in pancreatic juice.

In the present investigation these factors were studied by different methods. An autoradiographic technique was employed for determining the uptake of some intravenously injected, labelled amino acids in the pancreas of mice, rats and guinea pigs. This method affords possibilities of observing the uptake and disappearance of radioactivity in the pancreas as compared with other organs and tissues, and its distribution at varying times after the injection in different morphologic sections of the pancreas such as cells, acini, and excretory ducts.

The incorporation of S^{35}-methionine into pancreatic proteins was studied in mice and guinea pigs at various intervals after the injection. Its incorporation into the protein fraction and the occurrence of non-protein-bound radioactivity were compared in animals whose pancreatic secretory activity differed. Incorporation into the protein of different cell fractions was observed with special reference to the uptake by zymogen granules.

Another factor investigated was the rate at which labelled

proteins appeared in the pancreatic juice of cats following injection of labelled amino acids. The proteins were separated by paper electrophoresis, after which the distribution of radioactivity among the fractions was recorded.

Abbreviations; Definitions

CPM	counts per minute
mC	millicurie, $3.7 \cdot 10^7$ atomic disintegrations per sec.
μC	microcurie, $3.7 \cdot 10^4$ atomic disintegrations per sec.
mmole	millimole
TCA	Trichloroacetic acid
self-absorption (source in the sample)	number of retained particles / number of particles irradiated from all infinitely thin layers in the sample
specific activity	radioactivity per weight unit of radioactive material

CHAPTER I

Historical Survey

An exhaustive review of previous investigations into the synthesis and secretion of digestive enzymes in the pancreas will be found in monographs by BABKIN (1950) and THOMAS (1950) and in a paper communicated by JUNQUEIRA and HIRSCH (1956). No detailed historical survey will therefore be undertaken in this chapter, which will be concerned solely with papers that seem to be particularly relevant to the present study, as well as a few of the investigations reported in recent years.

HEIDENHAIN (1875) was one of the first to study the synthesis and secretion of enzymes in the pancreas. He observed the variations in stained preparations from dogs, and compared those from starved animals with others taken at varying intervals after feeding. He found that some resynthesis of zymogen granules occurred within 3–4 hours after the pancreatic secretion had been stimulated by feeding, but that the maximum restitution of granules did not occur until 14–16 hours had elapsed.—Numerous other workers later investigated, in fixed histologic preparations, the changes taking place in the cell during the secretory cycle (KÜHNE and LEA 1882, and others). COWELL (1928) who studied the pancreas of white mice *in vivo*, described the discharge of granules after injection of pilocarpine. HIRSCH (1932 a, b) elucidated with that method the rate at which the zymogen granules were resynthesized after having been discharged with pilocarpine; he observed that after 3–4 hours some granules had formed and had assumed their positions in the apical part of the cell. Other researchers duplicated and verified these observations in mice (DUTHIE 1933; RIES 1935).

VAN WEEL and ENGEL (1938) correlated morphologic with biochemical findings. They observed the restitution of zymogen granules and at the same time investigated the carboxypoly-

peptidase and dipeptidase activity in the pancreas. (Carboxypolypeptidase is a digestive enzyme occurring in the pancreatic juice, but dipeptidase is confined to pancreatic tissue.) They found that the content of the former enzyme fell to a minimum about three hours after stimulation of the pancreatic secretion with pilocarpine. Resynthesis to the pre-stimulation level then occurred after nine hours. Dipeptidase showed changes in the opposite direction.—KOMAROV, LANGSTROTH and MCRAE (1939) and LANGSTROTH, MCRAE and KOMAROV (1939) reported experimental and mathematical calculations designed to establish the time of resynthesis of proteins in the pancreatic juice. In their experimental studies, using secretin, they found that a two-hour interruption of secretory stimulation virtually sufficed for resynthesis. DALY and MIRSKY (1952) sought to establish the time required for resynthesis of lipase, protease, and amylase in mouse pancreas after stimulation of the secretion with pilocarpine and feeding. They noted complete resynthesis after six hours. Since the protein content of the pancreas was unchanged during the secretory cycle, they concluded that the discharge of enzymes coincided with a rapid synthesis of new precursor proteins which then underwent transformation into the characteristic pancreatic enzymes.

ALLFREY, DALY and MIRSKY (1953) and DALY, ALLFREY and MIRSKY (1955) were the first to employ isotope-labelled amino acids for studying the synthesis of proteins in the pancreas. The uptake of N^{15}-glycine was found to be high in the fraction containing the greatest amount of ribonucleoproteins (ALLFREY, DALY and MIRSKY 1953). In a later investigation, however, the uptake of N^{15}-glycine by trypsinogen and chymotrypsinogen in mouse pancreas proved to be higher than that in any of the ribonucleoprotein fractions studied (DALY, ALLFREY and MIRSKY 1955).

JUNQUEIRA, HIRSCH and ROTSCHILD (1955) observed maximum radioactivity in the proteins of the pancreatic juice 2 3/4 hours after intravenous injection of labelled glycine. They detected no radioactivity in the pancreatic juice after injection of labelled blood protein, and therefore considered that its proteins were completely synthesized by free amino acids.—Radioactivity in the pancreatic juice showed a similar behavior following injection

of other labelled amino acids (ROTSCHILD, HIRSCH and JUNQUEIRA 1957).

In vitro investigations designed to elucidate the synthesis of proteins in the pancreas have been reported in recent years. HOKIN (1951) and ULLMANN and STRAUB (1954, 1955) showed that when pancreas slices and homogenate were incubated in media containing amino acids, their amylase content rose. ULLMANN and STRAUB (1957) demonstrated that this rise of the amylase content in homogenate was probably an activation process and not a *de novo* synthesis.

CHAPTER II

Methods

Labelled Amino Acids

The labelled amino acids were supplied by Radiochemical Centre, Amersham, England; Abbot Laboratories, U. S. A., and Nuclear Carbide Company, U. S. A. Suppliers and specific activities are:

S^{35}-DL-Methionine (Amersham) 13.0–26.3 mC/mmole. DL-Phenylalanine-2-C^{14} (Nuclear Carbide Comp.) 0.311 mC/mmole. S^{35}-DL-Cystine (Amersham) 48.1 mC/mmole. S^{35}-L-Methionine (Abbot) 2.0 mC/mmole. Glycine-2-C^{14} (Amersham) 2.1 mC/mmole.

Experimental Animals

Mice, rats, guinea pigs and cats were used. The mouse pancreas is fairly diffuse but, on the other hand, its relative weight is rather high. Since the bulk of it is located along the spleen, it can be readily identified and dissected free. In the rat the pancreas is far more diffuse and has a number of lobes that ramify into the mesentery, so that it is difficult to separate from the fat. The guinea pig pancreas is less lobulated than in the rat and, moreover is readily localized along the spleen. In large guinea pigs weighing about 500 grams, the pancreas is enveloped by a considerable amount of fat, but in younger animals of about 300 grams there is little fat.—Mice were generally used for autoradiography; rats and guinea pigs, only in a few experiments.

The mice weighed about 20 grams, the guinea pigs usually around 300 grams, though with occasional variations between 200 and 400 grams, and the rats weighed about 150 grams. Mice and guinea pigs were suitable for experiments in Chapters V and VI with respect to the morphology and localization of the pancreas, and hence they were employed.

In order to secure a sufficient amount of pancreatic juice for the experiments in Chapters VII and VIII it was necessary to use larger animals than mice, rats and guinea pigs. Cats were found to be suitable, since they respond well to stimulation of the secretion with secretin (MELLENBY 1925; WILANDER and ÅGREN 1932 and others). The feline pancreas is divided into two well defined lobes. As a rule there are two excretory ducts, with their orifices separated by an interval of about 2 cm. The larger duct (duct of Wirsung) collects juice from both lobes and the two ducts passing from either lobe unite 1–2 cm from its entry in the intestine. Here the duct is short and broad, and passes into the duodenal papilla together with the bile duct. The other pancreatic duct (duct of Santorini) is small or may be absent. Its termination in the intestine is caudal to the duodenal papilla.

In the present experiments male and female cats, weighing 2.5–4.5 kg, were used.

Pancreatic Secretion in Normal Feeding, Starvation and Stimulation with Pilocarpine

A number of investigations have shown that when the pancreas is stimulated into discharging enzymes by uptake of food or by injection of cholinergic agents, the enzyme content of the tissue decreases. When the pancreas is no longer stimulated or when animals are starved, the acini accumulate zymogen granules and the enzyme content rises (BABKIN 1950).

To facilitate interpretation of the present findings the secretory behavior of the pancreas in mice and guinea pigs during starvation and on stimulation with cholinergic agents was studied in some detail. Pilocarpine was used as a cholinergic agent, since it has a powerful stimulatory effect on the secretion. Although it has been employed for that purpose in several experimental investigations (HIRSCH 1932; RIES 1935; DALY and MIRSKY 1952; and others), only incomplete data are available on the optimal dose and the duration of treatment.

For the purpose of investigating the secretory behavior of the

Table I. Amylase content in the pancreas of mice and guinea pigs. Each value indicates the mean from six animals. The amylase activity is recorded in milligrams hydrolyzed starch per milligram wet weight pancreas.

Treatment	Amylase activity	
	Mice	Guinea pigs
Normally fed	124	29
Starved for 24 hours	130	29
Starved for 48 hours	175	32

pancreas during normal feeding and starvation, and after injection of cholinergic agents, the amylase content of that organ was determined, as DALY and MIRSKY (1952) had done. It was found that mice and guinea pigs living under uniform conditions had a constant amylase content; also that feeding, and stimulation with cholinergic substances, reduced that content and hence afforded a criterion of the secretory state of the pancreas. The amylase content alone was determined, since the method in question was the simplest and fastest.—Secretion of the various digestive enzymes runs parallel (DALY and MIRSKY 1952), and hence determination of the amylase content provides a relative measure of the other digestive enzymes.

The starved animals had water *ad libitum*, and the mice that received the normal feed were kept in their cages until the start of the experiment. Pilocarpine hydrochloride in physiological saline was injected intravenously. Immediately after sacrifice of the animals the pancreas was removed and homogenized, and its amylase content was then determined as described on page 23.

The amylase content was studied in normally fed mice and guinea pigs and in others starved for one or for two days (table I).

In normally fed mice the amylase content was at the same level as that in mice starved for one day. In mice starved for two days, the content was higher.—This suggested that the secretory activity of the pancreas was the same in mice starved for one day as in the normally fed mice. If, on the other hand, the mice

Table II. Amylase content in the pancreas of mice and guinea pigs after intravenous injection of varying doses of pilocarpine hydrochloride. The animals were sacrificed 2 hours after the injection. The amylase activity is recorded in milligrams hydrolyzed starch per milligram wet weight pancreas. Each value indicates the mean from six animals.

Treatment	Amylase activity	
	Mice	Guinea pigs
Control	132	27
5 μg Pilocarpine hydrochl. per g of body weight	90	17
15 μg » » » » » » »	52	13
50 μg » » » » » » »	48	17
100 μg » » » » » » »	60	16

were starved for two days the secretion was evidently reduced, since the amylase content of the pancreas was higher.

The amylase content of guinea pig pancreas per milligram of wet weight was lower than in mice. When guinea pigs had been starved for one day the content was the same as that in normally fed counterparts, indicating that the secretory activity of the pancreas was unchanged at that time, just as in mice. On starvation of guinea pigs for two days the amylase content was at a higher level, this again being consistent with the findings in mice. However, there was only a slight difference in elevation of the amylase content between guinea pigs starved for one day and those starved for two days.

To elucidate the dosage of pilocarpine hydrochloride associated with maximum secretion in mice and guinea pigs, varying doses were injected. The animals were sacrificed after 2 hours, by which time the maximum discharge had occurred, as shown in figure I.

It is evident from table II that the maximum secretion in mice was obtained after injection of about 50 μg/g body weight. If the dose of pilocarpine was increased, the secretion was sometimes retarded. At a dosage of 100 μg/g the animals were greatly affected; some of them presented symptoms of intoxication and had

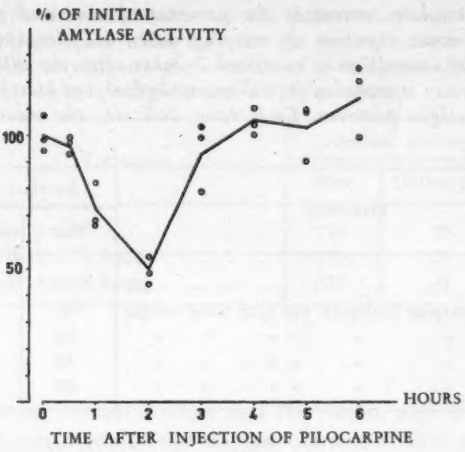

Fig. 1. Variation with time in the amylase content of mouse pancreas following intravenous injection of 50 μg pilocarpine hydrochloride per gram of body weight. Each circle indicates the value for combined samples from two animals.

profuse salivation and epiphora. The difference in elimination of amylase was very slight after injection of, respectively, 15 μg/g and 100 μg/g pilocarpine. Guinea pigs exhibited greater susceptibility to pilocarpine than mice; pronounced salivation and epiphora were already observed after injection of 15 μg/g body weight, and at larger doses the discharge of amylase from the pancreas decreased.

The duration of enzyme discharge after the pilocarpine injection was studied in mice (figure 1). Following intravenous injection of 50 μg pilocarpine per gram body weight the mice were sacrificed 30 minutes and 1, 2, 3, 4, 5 and 6 hours after the injection. Maximum discharge was found after 1 to 3 hours. The resynthesis of enzymes was pronounced; 4–6 hours after the injection the amylase content was higher than that in the controls.

Autoradiographic Technique

AUTORADIOGRAPHY OF WHOLE MICE SECTIONS

Experimental Animals

White mice of both sexes were used.

	Number of Animals
Distribution of S^{35}-DL-methionine	45
Distribution of S^{35}-L-methionine	9
Distribution of S^{35}-DL-cystine	28
Distribution of C^{14}-DL-phenylalanine	8
Distribution of C^{14}-glycine	4

Injections

All injections of labelled substances were given intravenously into a tail vein. The labelled amino acids were dissolved in physiological saline, and the injected volume amounted to 0.1–0.2 ml. Cystine, which is not readily soluble at pH 7, was dissolved at pH 2–3 by addition of hydrochloric acid to physiological saline; in these cases a volume of about 0.2 ml was injected.

Sectioning and Freeze-Drying of Whole Mice.

For studying the distribution of the labelled amino acids, a special autoradiographic method was employed (ULLBERG 1954). With this method whole sections of small animals can be autoradiographed and all organs observed on the same autoradiogram; moreover, release of isotopes from the slices is avoided. The mice were killed 5, 20, 30, 40 and 60 minutes and 2, 4, 6 and 24 hours after injection of methionine and cystine; or 5 minutes, 30 minutes, 1 hour and 4 hours after injection of phenylalanine and glycine.

Ullberg's technique was employed for all mice from which general autoradiograms were taken. The animals were killed in a mixture of carbon dioxide snow and acetone (— 70° C) at varying intervals after the injection. Their bodies were then transferred to a freezing chamber at — 10° C and mounted on a

specially designed object table of a sleigh microtome. A number of sagittal sections about 20 μ thick were subsequently taken with the aid of tape (PALMGREN 1954). The slices were dried in the freezing chamber for 2–3 days before being used for autoradiography.

Autoradiography

The films were exposed to whole sections of mice by apposition and subsequent separation. Gaevert Dentus Rapid film was used, and the slices were attached directly to the emulsion. A sheet of lead foil was placed on the back of each film, and batches of about six layers of sections and films were kept in presses. These latter were put into lightproof boxes and stored in a cold room at — 10° C. The exposure times varied from 5 to 15 days. The films were developed in G 230 for five minutes and fixed in G 305 A (Gevaert) at a temperature of 18–20° C. The contact autoradiograms were then studied under a microscope and compared with the sections from which they had been taken. To facilitate evaluation the slices were stained histologically in hematoxylin and eosin.

Quantitative Evaluation

Two methods were tried for obtaining quantitative values concerning the distribution of labelled substances in various organs and tissues.

1. Densitometric examination of autoradiograms.
2. Geiger-Müller measurement of pieces punched out of dried sections.

Densitometric determination has the disadvantage that a linear response is no longer obtained at very high degrees of density. On autoradiograms the pancreas shows far greater darkening than other organs; hence in this investigation the Geiger-Müller method was preferred for recording activity in the pancreas and evaluating it in relation to other organs. Circular pieces with an area of about 2 sq. mm were punched out of the dried slices, then placed on aluminium dishes and the radioactivity measured in a Geiger-Müller tube. Radiation absorption in the tissues

was negligible, since the weight of the slices was less than 1 mg per square centimeter. The results were plotted on a curve showing variations in radioactivity for a number of organs at different times after injection.

AUTORADIOGRAPHY OF THE PANCREAS

Experimental Animals

White mice, white rats, and guinea pigs were used.

	Animals	Number
Distribution of S^{35}-DL-methionine	Mice	28
	Rats	12
	Guinea pigs	6
Distribution of S^{35}-DL-cystine	Mice	12
	Rats	8

Injections

All injections were intravenous, and the labelled amino acids were dissolved in physiological saline. A volume of 0.1–0.2 ml was injected. With mice the injections were made in a tail vein; with rats, in the femoral vein under light ether anesthesia; with guinea pigs, in a cubital vein. In the two last-named animals an incision was made in the skin to facilitate the injection.

Histologic Technique

The animals were sacrificed by decapitation 15 minutes, 30 minutes, and 1, 4, 6 and 24 hours after the injections. The pancreas was removed and pieces about 2 mm in size were either fixed in acetic acid and ethanol or freeze-dried. Freeze-drying was done in a modified Glick-Malmström apparatus (MOBERGER, LINDSTRÖM and ANDERSSON 1954) and proceeded for 12 hours. The method recommended by the latter authors was used for embedding in paraffin. The embedded preparations were then cut into sections about 5 μ thick and mounted on object glasses, without floating on water. Some of these slices were deparaffinized in xylene-ethanol for use in stripping-film autoradiography.

Parts of the pancreas were also fixed in acetic acid and ethanol (1:3), then embedded in paraffin and cut into sections about 5 μ thick, which were mounted on object glasses. The slices were subsequently deparaffinized in xylene-ethanol.

Autoradiography

Two methods were employed.

Apposition technique. Freeze-dried sections which had not been deparaffinized were used for apposition autoradiography by the method described in the foregoing (page 18).

Stripping film technique. Histologic sections from pancreas, freeze-dried and fixed in acetic acid and ethanol, were treated largely by the method of PELC (1947). Before application to the film, the sections were deparaffinized in xylene and passed through absolute alcohol, 70 per cent alcohol, and distilled water. Eastman Kodak autoradiographic stripping film was employed. Before application of the sections, object glasses were treated with chrome alum gelatin *ad modum* BOYD (1955).

When the sections had been covered with emulsion they were dried in air, a fan being used to hasten drying. The exposure time ranged from 15 days to 3 months. The autoradiograms were then developed in Kodak D 19 for 5 minutes at 18–20° C and rinsed in water for 10 seconds. Thereafter they were fixed in Kodak F 5 for 5–10 minutes and rinsed in running water for 30 minutes. The sections were stained in hematoxylin and eosin, and they and the stripping film were mounted in Canada balsam. The slices were stained through the emulsion, and for this reason the film took up some dye, though not sufficiently to affect the transparency. Some sections were left unstained for examination under a phase contrast microscope.

Evaluation of Autoradiograms

Apposition Autoradiography. The density was evaluated visually in the sections. No comparison was made of the density of sections taken at different times after the injection.

Stripping Film Method. The Eastman Kodak stripping film employed here is fine grained and has an emulsion layer of 5 μ.

Evaluation was based on visual observation of the grain density over different cells and parts thereof.

Resolving Power

Autoradiograms taken with Gaevert Dentus Rapid film have a low resolving power which may be estimated at about 20 μ and does not permit a study of single cells. Stripping film has a far greater resolving power. PELC (1957) calculated a resolving power of 1–2 μ under ideal conditions and with the use of soft beta-emitters as C^{14} and S^{35}.

Determination of the Radioactivity in the Protein Fraction and in the Trichloroacetic Acid (TCA) Soluble Fraction from Pancreatic Tissue and Pancreatic Juice

MEASUREMENT OF THE RADIOACTIVITY IN PANCREATIC TISSUE

Homogenization

The animals were sacrificed by a blow on the head and exsanguinated by decapitation. The pancreas was immediately removed and cooled in iced physiological saline, then homogenized *ad modum* POTTER and ELVEHJEM (1936) at 0° C.

Isolation of protein for radioactivity measurement

The homogenized pancreatic tissue or pancreas cell fractions were treated mainly *ad modum* SCHNEIDER (1945). In all experiments the radioactivity in the total proteins was determined. The protein was precipitated by adding to the homogenate an equal volume of iced 10 per cent TCA, followed by centrifugation at 3,000 *g* for 30 minutes. The supernatant was taken for measurement of radioactivity, and the sediment was washed three times in 5 per cent TCA, with addition of inactive methionine in the

second wash. The sediment was then heated in 5 per cent TCA at 90° C and centrifuged; after which it was washed once in warm 95 per cent ethanol, twice in ethanol-ether 1:1, and once in ether. Thereafter, the protein was suspended in ether and dried and ground.

Counting

In the TCA-soluble fraction the radioactivity was determined by taking 0.05 ml from the filtrate and the first wash obtained after precipitation of the proteins with TCA, and allowing the fluid to evaporate in stainless steel dishes. The radioactivity in the second and third wash was not determined, since the latter had been found, in several experiments, to contain only 1 per cent or so of the amount present in the filtrate after precipitation of the proteins.

The radioactivity of the precipitate was determined by spreading the pulverized protein on aluminium dishes with an area of 1 square centimeter. The samples were counted with a thin mica window Geiger-Müller counter (window thickness 1.8 mg/cm^2). A correction was made for self-absorption of the dishes by conversion according to a self-absorption curve that had been plotted for protein containing S^{35}.

MEASUREMENT OF THE RADIOACTIVITY IN PANCREATIC JUICE

From every sample of pancreatic juice 0.05 ml was taken and evaporated to dryness on aluminium dishes. An arrangement according to RYDBERG (1958) was used while drying the samples. In this way reproduceable values were obtained. The pancreatic juice was dried on such a large surface that the weight of the dried substance was less than 1 mg per cm^2. Correction for self-absorption thus could be omitted (KAMEN 1955).

The radioactivity of the protein bound and the TCA soluble fractions in pancreatic juice was estimated. To a sample of pancreatic juice an equal volume of an ice-cold 10 per cent TCA solution was added in order to precipitate the proteins.

Isolation of protein and counting was then done according to the method described on pages 21 and 22.

The samples were counted with a thin mica window Geiger-Müller counter (window thickness 1.8 mg/cm^2).

Enzyme Assays

Amylase

Amylase was assayed by the method of SMITH and ROE (1949, 1957), which is based on the hydrolyzing power of the enzyme on starch. The enzyme activity was recorded in milligrams of hydrolyzed starch. One milliliter was taken from the pancreas homogenates and diluted in physiological saline to one part in 500–2,000, depending on the expected enzyme activity. To ascertain if this method of determining the amylase content yielded duplicable values, a sample of 1 g guinea pig pancreas was taken and homogenized. Twenty samples were then taken from the homogenate and each of them was assayed for amylase. The mean and standard deviation amounted to 26 $\pm$ 1.8 mg hydrolyzed starch per 1 mg wet weight. This result demonstrated that the method yielded fairly duplicable values. To reduce the chances of wrong values, however, duplicate determinations were invariably made and the mean recorded.

Lipase

Lipase was determined by the method reported by SELIGMAN and NACHLAS (1950), which is based on the hydrolysis of β-naphthyllaurate by lipase, whereby β-naphthol is formed. β-napthyllaurate was prepared according to NACHLAS and SELIGMAN (1949). The enzymatic activity was recorded as the amount of liberated β-naphthol.

Proteolytic Activity

Proteolytic activity was determined *ad modum* ANSON (1938), whose method is based on the breakdown of hemoglobin by the

proteolytic enzymes. The latter were activated with enterokinase. (The hemoglobin was kindly supplied by Dr. B. Olsson, Kungl. Veterinärhögskolan.) The enzymatic activity was recorded as the amount of liberated tyrosine.

Trypsin

Trypsin was assayed *ad modum* SCHWERT and TAKENAKA (1955). Benzoyl-arginine and N-benzoyl-arginine ethyl ester, synthesized according to BERGMANN, FRUTON and POLLOK (1939), were used for the determination.

Chymotrypsin

Chymotrypsin was assayed *ad modum* SCHWERT and TAKENAKA (1955). L-tyrosine (HOPKINS and WILLIAMS) and tyrosine ethyl ester hydrochloride (Eastman Kodak) were used for the determination.

Activation of Trypsinogen and Chymotrypsinogen

Of the pancreatic juice enzymes, trypsin and chymotrypsin are excreted in inactive forms, trypsinogen and chymotrypsinogen. It is accordingly necessary to activate them prior to enzyme assay.

In order to activate trypsinogen, enterokinase (prepared according to KUNITZ 1939 and kindly supplied by Dr. B. Olsson, Kungl. Veterinärhögskolan) was added to the pancreatic juice solution, after which the latter was stored at 0° C for 24 hours. For activating chymotrypsinogen, crystalline trypsin (Worthington) was added to the pancreatic juice solution, after which the latter was stored at 0° C for 24 hours.

Measurement of Total Protein

Total protein was determined *ad modum* LOWRY *et al.* (1951). A standard curve was secured by parallel nitrogen determination according to Kjeldahl's micromethod *ad modum* NIDERL and NIDERL (1952). In measurement of the protein content of pancreatic juice the conversion factor of 6.25 was used.

Separation of Various Cell Fractions of the Pancreas and Determination of the Enzymatic Activities in the Isolated Fractions

Homogenization

Following decapitation of the animals, the pancreas was removed and immediately cooled in iced physiological saline. It was thereafter homogenized *ad modum* POTTER and ELVEHJEM (1936). Homogenization was done in 0.25 M sucrose at 0° C.

Centrifugation

Following homogenization of the pancreatic tissue the homogenate was filtered through plankton cloth to eliminate detritus of connective tissue. Cell fractions were isolated by means of differential centrifugation in 0.25 M sucrose, largely by the method of HOGEBOOM, SCHNEIDER and PALADE (1948) and, for zymogen granules, by the method of HOKIN (1955), at a temperature of 0° C. The final dilution of the homogenate was regularly adjusted to 1:10 (tissue weight:final homogenate volume). Pancreatic tissue from two or three animals was used in each experiment.

1. The *nuclear fraction* was obtained by centrifugation of the original homogenate for 10 minutes at 500 *g*.

2. The *zymogen granule fraction* was separated after centrifugation of the supernatant of *1* for 10 minutes at 1,000 *g*.

3. The *mitochondrial fraction.*—The supernatant of *2* was centrifuged for 10 minutes at 2,000 *g*. The sediment was discarded and the supernatant centrifuged at 8,000 *g* for 10 minutes. The resulting sediment constituted the mitochondrial fraction.

4. The *microsomal fraction* was separated by centrifugation of the supernatant of *3* for 55 minutes at 105,000 *g*.

5. The *supernatant fraction* was the supernatant of *4*.

An International refrigerator centrifuge was used for separating the nuclear, zymogen and mitochondrial fractions; a Spinco model L ultracentrifuge for the microsomal fraction. The resulting fractions were washed once by suspension in 0.25 M sucrose solu-

tion, then centrifuged at the same gravity at which they had been separated. Each fraction was checked by examination of smears; unstained ones were studied under a phase contrast microscope; those stained with hematoxylin and eosin, under a light microscope.

THE ISOLATED FRACTIONS

Nuclear Fraction

This fraction was obtained after centrifugation for 10 minutes at 500 *g*. It was not purely nuclear but contained some other cell particles. Quite a number of cytoplasmic fragments were attached to the nuclei, and small particles resembling the zymogen granules were fairly abundant in that fraction. Other cellular debris that might have been detritus of connective tissue and excretory ducts was also observed. In short, this fraction may be described as fairly heterogeneous.

The Zymogen Granule Fraction

This fraction was obtained after centrifugation for 10 minutes at 1,000 *g*. The fraction appeared to be relatively pure. The zymogen granules were discernible as spherical homogeneous bodies with diameters of about 0.5–1.5 μ. The smear nevertheless showed, here and there, some granular elements that presumptively were large mitochondria.

The Mitochondrial Fraction

The fraction obtained on centrifugation at 2,000 *g* contained a few zymogen granules and some granuliform elements that probably were mitochondria. This fraction was usually discarded; the aim of centrifuging at 2,000 *g* was to eliminate any zymogen granules that had not sedimented at 1,000 *g*.

The fraction that was termed the mitochondrial fraction and had resulted from centrifugation at 8,000 *g* for 10 minutes contained granuliform particles which were regarded as mitochondria. This fraction was virtually devoid of zymogen granules.

The Microsomal Fraction

This was obtained after centrifugation at 105,000 *g* for 55 minutes. The sediment appeared to be homogeneous and was of a clear light red color.

On the whole, the fractions secured by the above procedure could not be regarded as cytologically homogeneous. In particular, this was true of the nuclear fraction, though the zymogen granule fraction too, which appeared to be the purest, contained other particles. However, the bulk of each cell fraction doubtless consisted of those cellular components that have been designated as such.

DISTRIBUTION OF AMYLASE, LIPASE AND PROTEOLYTIC ACTIVITY IN THE CELL FRACTIONS

In the present study it was decided to investigate the distribution of amylase, lipase and proteolytic activity, since they are the principal enzymes in the pancreatic juice.

In table III are detailed amylase, lipase and proteolytic activity in pancreas from guinea pigs starved for 24 hours and in others starved for 24 hours and then injected with 15 μg pilocarpine hydrochloride per gram of body weight 90 minutes before being sacrificed.

In starved animals with abundant zymogen granules in the pancreas, the amylase and proteolytic activity was found to be substantially higher in the zymogen granule fraction than in any other fraction.

This finding was in agreement with the results reported by HOKIN (1955), though he isolated only zymogen granules and compared them with the total homogenate. LAIRD and BARTON (1957) and SIEKEVITZ and PALADE (1958 a), too, observed that amylase and trypsin were concentrated to zymogen granules. On the basis of the present and earlier results, it may be assumed that the digestive enzymes produced in the pancreas are localized in the zymogen granules before being excreted.

Pilocarpine-treated and untreated guinea pigs that had been starved for 24 hours were subjected to a comparative investiga-

Table III. Distribution of amylase, lipase and proteolytic activity in the pancreatic cell fractions of guinea pigs starved for 24 hours, with and without subsequent pilocarpine treatment. Each value indicates the mean from four guinea pigs.

Cell Fraction	Amylase mg hydrolyzed starch per mg wet weight pancreas		Lipase mg liberated beta-naphthol per mg wet weight pancreas		Proteolytic activity mmole × 10^6 tyrosine per mg wet weight pancreas	
	Starved and pilocarpine treated	Starved	Starved and pilocarpine treated	Starved	Starved and pilocarpine treated	Starved
Nuclear fraction ...	2.1	2.3	70	55	5.2	5.9
Zymogen granule fraction	3.1	8.1	40	50	6.3	10.1
Mitochondrial fraction ...	1.6	1.7	70	125	2.1	4.7
Microsomal fraction ...	2.4	2.1	340	338	6.7	5.3
Supernatant fraction ...	4.6	5.8	883	1,029	0	0

tion of the protein and the amylase content of the zymogen granule fraction (table IV). That fraction was found to be about twice as great in the untreated animals.

The amylase content was considerably higher in the zymogen granule fraction than in other fractions in starved guinea pigs, but after stimulation of the secretion it was low. The supernatant fraction had a fairly constant, high amylase content which did not vary in the two groups. In the microsomal fraction the amylase content was somewhat higher in pilocarpine-stimulated than in starved animals.

The lipase distribution differed from the amylase and proteolytic activity in that no concentration of lipase activity was found

Table IV. Comparison of protein content and amylase content of the zymogen granule fraction in guinea pigs starved for 24 hours, with and without subsequent pilocarpine treatment. Each value indicates the mean from three guinea pigs.

Treatment	mg protein-N per g wet weight pancreas	mg hydrolyzed starch per mg wet weight pancreas
Starved guinea pigs	2.6	6.3
Starved and pilocarpine-treated guinea pigs	1.3	1.9

in the zymogen granules (table III). The bulk of lipase was localized in the supernatant and microsomal fraction. There was no conspicuous difference in lipase distribution following stimulation of the secretion with pilocarpine.

The proteolytic activity had a far more heterogeneous distribution than lipase and amylase. The supernatant, which contained amylase and lipase in abundance, had no proteolytic activity. HOKIN (1955) demonstrated that supernatant contained a trypsin inhibitor. The high content of proteolytic activity in the zymogen granule fraction in starved animals was closely consistent with the distribution of amylase. There was an insignificant difference between starved and pilocarpine-treated guinea pigs with respect to proteolytic activity in the microsomal fraction.

The observed heterogeneity in distribution of the enzymes might be due to localization of the various digestive enzymes in separate granules. The granules that contain proteolytic activity and amylase might be broken down at homogenization, so that the lipase activity is found chiefly in the supernatant.

Paper Electrophoresis

Apparatus

Paper electrophoresis was done with a horizontal apparatus made of plastic *ad modum* DETTKER and ANDURÉN (1954).

Buffer

Veronal buffer with pH 8.6 and the ionic strength 0.1 according to MICHAELIS (1931) was employed.

Paper

Whatman no. 1 filter paper was found to be best suited for these experiments. It absorbed the least material and dye, and discoloration of the background was lighter than that with other Whatman papers tested.

Procedure

Most samples of pancreatic juice had a protein content of between 0.5 and 2 g per 100 ml. If the protein content was less than 1 g per 100 ml the juice was concentrated in a freeze-drying apparatus for histologic preparation *ad modum* MOBERGER, LINDSTRÖM and ANDERSSON (1954). Two milliliters of juice was treated, and the dried mass was then dissolved in 0.5 ml of the same pancreatic juice.

As a rule 0.05 ml of natural pancreatic juice and 0.03 ml of the freeze-dried juice were each placed on 5 cm wide strip. The paper was moistened with buffer solution and the excess fluid was absorbed between filter papers. The pancreatic juice was applied with a special micropipet and ruler (Kelab AB, Stockholm) in a straight line perpendicular to the direction of movement. A constant voltage of 180 V, giving a current strength of about 0.2 mA, per strip was used. The electrophoresis was performed for 20 hours at $+20^{\circ}$ C.

Staining for Protein Determination

The strips were dried at 80° C and stained with amido black 10 B *ad modum* GRASSMANN, HANNIG and KNEDEL (1951). Excess dye was removed by washing the strips in a solution of 3 per cent phenol in 10 per cent (vol/vol) acetic acid (GRASSMANN and HANNIG 1952).

Determination of the Color Intensity on the Paper Electrophoretic Strips

The color intensity on the paper strips was measured *ad modum* GRASSMANN, HANNIG and KNEDEL (1951) in a Spinco Analytrol Recording Scanner and Integrator (Beckman Inc., U. S. A.).

Distribution of Digestive Enzymes on the Paper Electrophoretic Strips

Paper electrophoretic strips used for localizing the enzyme were dried with a fan at room temperature, which process took a few minutes. The strips were then cut into halves, one of which was stained with amido black 10 B for localization of the protein fractions, and the other was cut into 0.5 cm wide pieces, which were used for assay of amylase, lipase, total proteolytic activity, trypsin and chymotrypsin, after eluation in physiological saline for 20 hours at 0° C.

Determination of Radioactivity on the Paper Electrophoretic Strips

The radioactivity on the strips was determined by making autoradiograms with Kodak Industrex (X-ray) film. The exposure time ranged from three weeks to six months, and the films were developed in G 305 (Gaevert). After exposure the strips were stained. In a few instances, especially in the case of using C^{14}-DL-phenylalanine, pieces measuring about 0.5 sq. cm were cut from the electrophoretic strips and the radioactivity therein determined in a Windowless Flow Counter (Tracerlab, U. S. A.).

CHAPTER III

Uptake and Disappearance of Radioactivity in the Pancreas in Relation to Other Organs Following Intravenous Injection of Labelled Amino Acids

This part of the investigation was conducted in collaboration with S. Ullberg.

Several autoradiographic studies have been concerned with the distribution of labelled amino acids among various organs (BELANGER 1956; FICQ and BRACHET 1956; LEBLOND, EVERETT and SIMMONS 1957), and they have shown a high uptake of amino acids in tissues that produce protein for secretion, as for instance pancreas and intestinal mucosa. However, they have not dealt with variations in radioactivity in different organs shortly after the injection, when the bulk of the labelled amino acids is incorporated into protein (HULTIN 1950; BORSOOK *et al.* 1950).

In the present investigation the distribution of some labelled amino acids which had been injected intravenously was studied with special reference to the uptake and variations of radioactivity in the pancreas at varying intervals after the injection.

The distribution was studied by means of autoradiography and using the methods described on pages 17 and 18. White mice received an intravenous injection of 0.5 μC S^{35}-DL-methionine or S^{35}-DL-cystine, corresponding to 2–5 μg, per gram of body weight. In using C^{14}-glycine and C^{14}-DL-phenylalanine a dose of 0.2 μC per gram of body weight, equivalent to 7 μg and 106 μg respectively, was injected.

RESULTS

Autoradiograms showing the distribution of radioactivity at various times after injection of methionine (figures 2 and 3), and the results of quantitative measurements of pieces of sections in

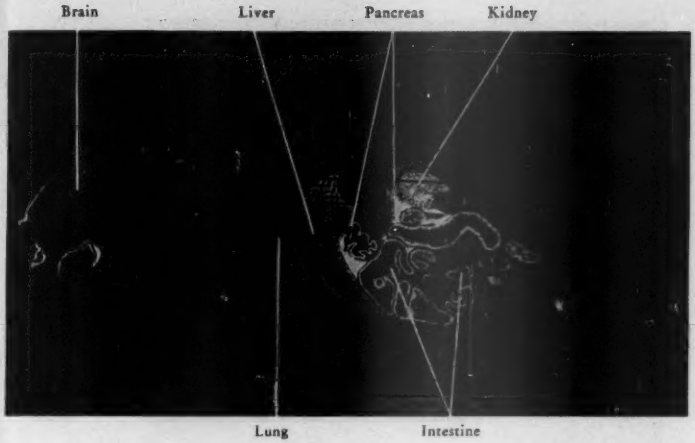

Fig. 2. Autoradiogram showing the distribution of radioactivity in a mouse 5 minutes after intravenous injection of S^{35}-DL-methionine. White areas correspond to high radioactivity. Most of the radioactivity has already left the blood. Accumulation can be seen in the pancreas, intestine, liver and kidney. Gaevert Dentus Rapid. Exposure time: 10 days. ×1.5.

Geiger-Müller tubes (figure 4), demonstrate that the bulk of the intravenously administered methionine rapidly leaves the blood and is taken up by the tissues.

Figure 2 shows the distribution five minutes after the injection. The pancreas already had a concentration several times greater than that in the blood, which was also exceeded by that in the liver, intestinal mucosa, kidneys, spleen, salivary glands, and bone marrow.

Thirty minutes after the injection (figure 3) the concentration in the blood had fallen further and that in the pancreas had reached its maximum. Otherwise the distribution was very similar to that after 5 minutes.

The curve in figure 4 shows how the distribution of radioactivity changed during the first six hours after the injection. The pancreas had by far the highest content of radioactivity, which also decreased relatively far more rapidly than in other

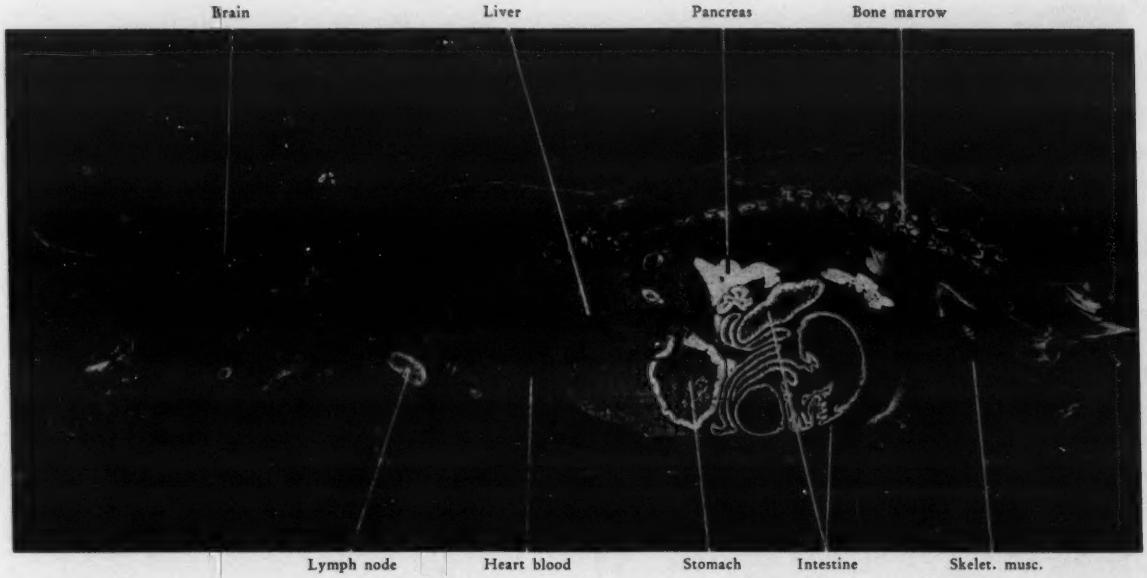

Fig. 3. Autoradiogram showing the distribution of radioactivity in a mouse 30 minutes after intravenous injection of S^{35}-DL-methionine. Note high activity in pancreas, gastric mucosa, intestinal mucosa, liver, lymph nodes and bone marrow. Gaevert Dentus Rapid. Exposure time: 10 days. ×2.5.

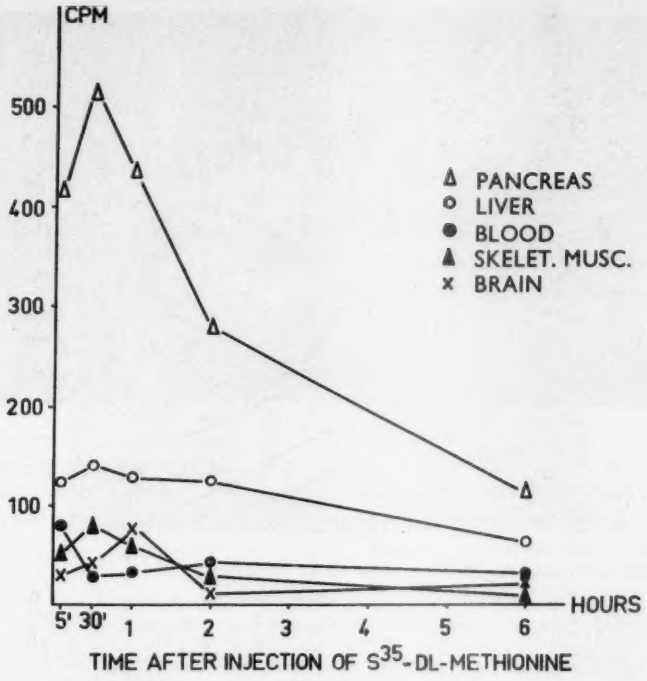

Fig. 4. Variation with time in the relative radioactivity of some organs of mice following intravenous injection of S^{35}-DL-methionine. Circular pieces were punched out of the dried sections of whole mice and the radioactivity was measured in a Geiger-Müller tube. Each value indicates the mean from two animals.

organs. Six hours after the injection it approached the content in the other organs, and after 24 hours (figure 5) it was only slightly higher than that in the muscles.

The radioactivity in the blood showed no further fall after 30 minutes, but rather tended to rise.

On autoradiograms the gastric and intestinal mucosa was second only to the pancreas in its concentration of radioactivity. It was too thin to be included in Geiger-Müller determinations.

methionine. Note high activity in pancreas, gastric mucosa, intestinal mucosa, liver, lymph nodes and bone marrow. Gaevert Dentus Rapid. Exposure time: 10 days. ×2.5.

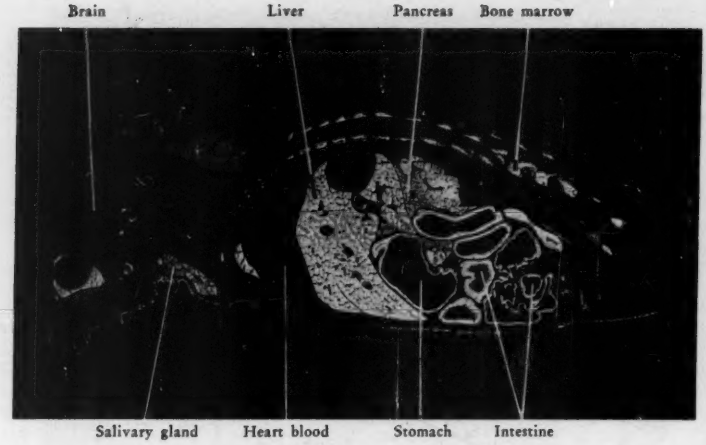

Fig. 5. Autoradiogram showing the distribution of radioactivity in a mouse 24 hours after intravenous injection of S^{35}-DL-methionine. There is high activity in liver, intestine and bone marrow, relatively high activity in salivary glands and pancreas.
Gaevert Dentus Rapid. Exposure time: 15 days. ×1.5.

Initially after the injection the radioactivity in the gastric mucosa (figure 6) was concentrated to the zymogen cells, but later on it showed a more even distribution throughout the mucosa.

The salivary and lacrimal glands exhibited a much lower concentration of radioactivity than the pancreas.

Excretion of radioactivity into the urine was detectable. Only five minutes after the injection radioactivity was present in the renal pelvis and urinary bladder. It was also observed in the gastric and intestinal contents at varying times after the injection, though chiefly after one hour or more. The distribution of S^{35}-DL-methionine was compared with the distribution of S^{35}-DL-cystine, C^{14}-DL-phenylalanine, and C^{14}-glycine.

The other amino acids that were studied behaved similarly to methionine with regard to their accumulation in the pancreas in relation to other organs and the times of their uptake and disappearance. Figures 7 and 8 illustrate the distribution of,

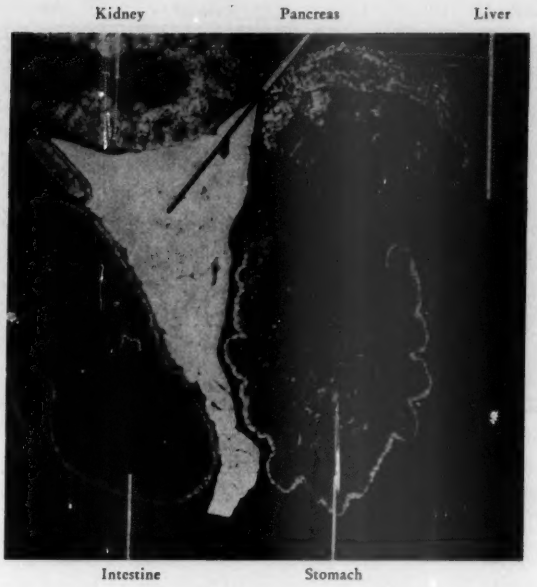

Fig. 6. Autoradiogram showing the distribution of radioactivity in a mouse 30 minutes after intravenous injection of S^{35}-DL-methionine. Note high activity in pancreas, zymogen cells of gastric mucosa, intestinal mucosa and kidney.
Gaevert Dentus Rapid. . Exposure time: 10 days. ×6.

respectively, S^{35}-cystine and C^{14}-phenylalanine 30 minutes after intravenous injection.

In other respects too, the distribution pattern of the different amino acids was, on the whole, similar. Cystine and glycine differed from the others in that their passage to the central nervous system was to some extent barred. Otherwise there was some specific localization such as an exceptionally high incorporation of the sulfur-bearing amino acids into hair. Following injection of S^{35}-cystine, substantial radioactivity was observed in cartilage and blood-vessel walls, but this was not the case with S^{35}-methionine.

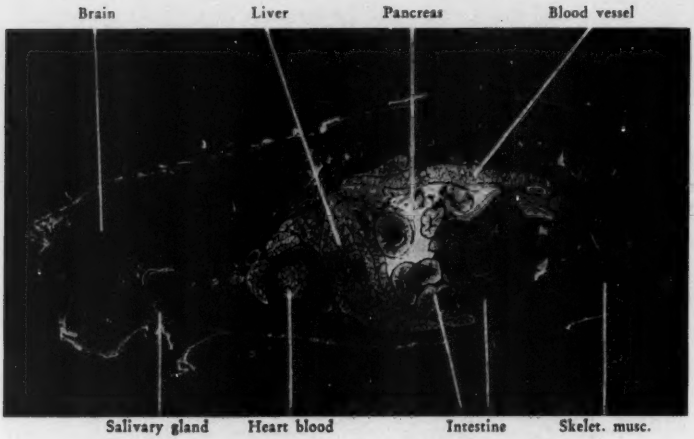

Fig. 7. Autoradiogram showing the distribution of radioactivity in a mouse 30 minutes after intravenous injection of S^{35}-DL-cystine. Note high activity in pancreas.
Gaevert Dentus Rapid. Exposure time: 10 days. ×1.5.

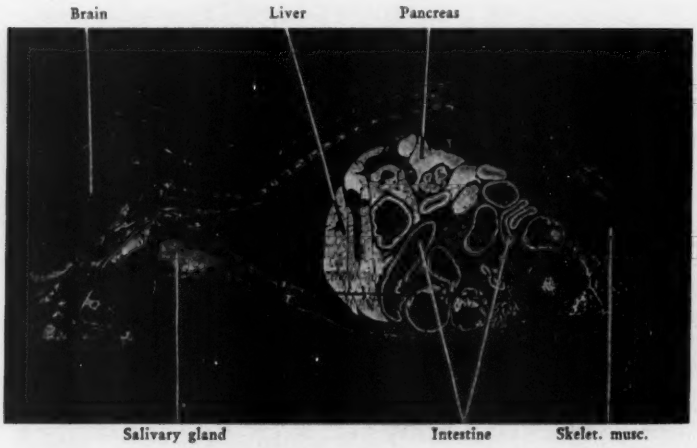

Fig. 8. Autoradiogram showing the distribution of radioactivity in a mouse 30 minutes after intravenous injection of C^{14}-DL-phenylalanine. Note high activity in pancreas, intestine and salivary glands.
Gaevert Dentus Rapid. Exposure time: 20 days. ×1.5.

DISCUSSION

The method employed for studying the distribution of labelled amino acids in sections of whole mice did not involve release of the acids, and it enabled their uptake by different organs to be compared and considered in relation to that by the blood. Hence the method was well suited for investigating the absorption of amino acids from the blood. It was striking to observe the swiftness with which they left the blood stream and were taken up by various organs and tissues.

Since the pioneering investigations by SCHOENHEIMER (1942) and co-workers it has been apparent that the body proteins are constantly and rapidly degraded in whole or in part and as rapidly reconstituted.

SCHOENHEIMER and his co-workers fed adult rats with N^{15}-labelled amino acids and found that different tissues take up labelled amino acids into their proteins at quite different rates, so that when examined at a short interval after cessation of administration the specific activities of the proteins of different tissues are found to be widely divergent. These and more recent investigations carried out by administering various labelled amino acids have led to rather similar results (NIKLAS *et al.* 1958 and others). The tissues which rapidly attain high radioactivity are intestinal mucosa, liver, pancreas, spleen and bone marrow. Other tissues like muscle, brain and skin attain low activities.

Earlier investigators (FRIEDBERG and GREENBERG 1947; LIDSTRÖM 1954) have shown that the amino acid content in blood rapidly falls after intravenous injection of amino acid mixtures or protein hydrolysates. They found a high uptake of amino acids in liver and kidney and a lower uptake in skeletal muscles.

The present investigation has shown that the amino acids very soon leave the blood and accumulate in certain organs, especially the pancreas. The pancreas has been found to accumulate the different amino acids to a greater extent than any other organ. The swift uptake by the organs points to a rapidly acting receptor mechanism for the cellular uptake of amino acids transported by the blood.

The proportional concentrations of various amino acids in

different organs were very similar in different animals for the same survival time. The distribution of radioactivity was very similar from 5 minutes to about 4 hours after the injection. It then changed, chiefly because the glands discharged their secretion but the content of radioactive substance persisted longer in cytogenous tissues such as bone marrow, spleen, lymph glands and thymus; it remained longest and most evenly in those tissues which produce neither secretion nor cells but presumably show an uptake mainly by breakdown of old and formation of new peptide bonds in structural proteins (e. g. muscles, lungs, compact bones).

The intestine produces not only secretion but also cells. The labelled secretion originating from the pancreas may perhaps, later on, influence the concentration curve for the intestinal mucosa and contribute to its more prolonged course in relation to that for the pancreas.

The slight secondary increase in the radioactive content of the blood (figure 4) may be attributable to the supply of blood proteins produced in the liver (MILLER and BALE 1954).

The lower concentration of amino acids in e. g. salivary and lacrimal glands in relation to the pancreas may be chiefly due to the fact that they have less extensive synthesis of proteins than the pancreas.

A possible source of error in the use of labelled amino acids as precursors for studying the synthesis of proteins is that the labelling substance may form another chemical compound instead of becoming an amino acid component of protein. Transformation of methionine into cystine has been demonstrated by TARVER and SCHMIDT (1939), DU VIGNEAUD (1952), and others. Since, however, cystine is probably incorporated into protein on the same scale as methionine, this metabolic transformation is unlikely to have any major significance. The labelled sulfur in methionine has been recovered in inorganic form (DU VIGNEAUD 1952), but this metabolic process is presumably a minor one, and the present autoradiograms did not show uptake of S^{35} by cartilage and connective tissue, as is the case after injection of inorganic sulfur (ODEBLAD and BOSTRÖM 1952). It may nevertheless be observed following injection of S^{35}-cystine, which suggests

that the S^{35} from cystine has been incorporated into connective tissue and cartilage.

The amino acids used in the present investigation were chiefly the racemic (DL) form. In the organism only the L-form of amino acids is utilized in the synthesis of protein. However, growth has been observed in rats whose feed contained only the D-form (WRETLIND 1950, 1952 a, b). This shows that the D-form can be utilized too, but it will require stereo-naturalization in the organism. The extent and rate of this process in different tissues are unknown.

Labelled amino acids are not only incorporated into pancreatic secretion products; they may be assumed to be incorporated also into structural proteins of the pancreas. The minor significance of this in comparison to the high radioactivity of the secretion products is shown, however, by the swift decrease in pancreatic radioactivity when the organ discharges its contents. This source of error can be partially eliminated, especially in stripping film autoradiography (Chapter IV), for the secretion products may be traced as far as the acinar lumina and the ducts.

CHAPTER IV

Distribution of Radioactivity in the Pancreas at Varying Times after Intravenous Injection of S^{35}-DL-Methionine and S^{35}-DL-Cystine

The autoradiographic investigations of BELANGER (1956), FICQ and BRACHET (1956), and LEBLOND, EVERETT and SIMMONS (1957) show that the exocrine part of the pancreas has a high uptake of labelled amino acids. These authors did not, however, study the distribution of radioactivity in the pancreas at varying times after the injections.

In the present section it was sought to ascertain where the radioactivity taken up by the pancreas was localized at different intervals, in order to find out when it was present in the zymogen granules and when it was discharged through the excretory ducts.

By means of autoradiography and using the methods described on pages 19 and 20, the distribution of S^{35}-DL-methionine and S^{35}-DL-cystine in the pancreas was studied. White mice, white rats and guinea pigs received an intravenous injection of 0.5 μC S^{35}-DL-methionine or S^{35}-DL-cystine, corresponding to 2–5 μg, per gram of body weight.

RESULTS

The results reported below derive from a material consisting of apposition autoradiograms from freeze-dried pancreas and stripping film autoradiograms from such pancreas as well as pancreas fixed in acetic acid and ethanol.

Stripping film autoradiograms of freeze-dried sections had a higher degree of density than those obtained with chemically fixed sections, though there was no appreciable difference in the distribution of radioactive substance. In reporting the results from the stripping film material no distinction is made, therefore, between the two groups.

Fig. 9. Autoradiogram and a 5 μ section of mouse pancreas showing the distribution of radioactivity 60 minutes after intravenous injection of S^{35}-DL-methionine. The black spots (silver grains) indicate deposition of radioactivity. Stripping film autoradiography was used. Note high activity in the exocrine part but low activity in the islands of Langerhans and the connective tissue. Eastman Kodak stripping film. Exposure time: 42 days. ×120.

On autoradiograms of sections taken during the first few hours after the injections there was a high concentration of radioactivity in the exocrine part of the pancreas but little or no radioactivity in the islands of Langerhans. This pattern applied to both S^{35}-methionine (figure 9) and S^{35}-cystine.

No definite concentration of radioactivity in any part of the excretory cells was detectable in the first 30 minutes after injection, but the activity was greater in the basal part of the cells than in the centers of the acini. That in the excretory ducts was low 15 and 30 minutes after the injection.

Autoradiograms one hour after injection showed a different

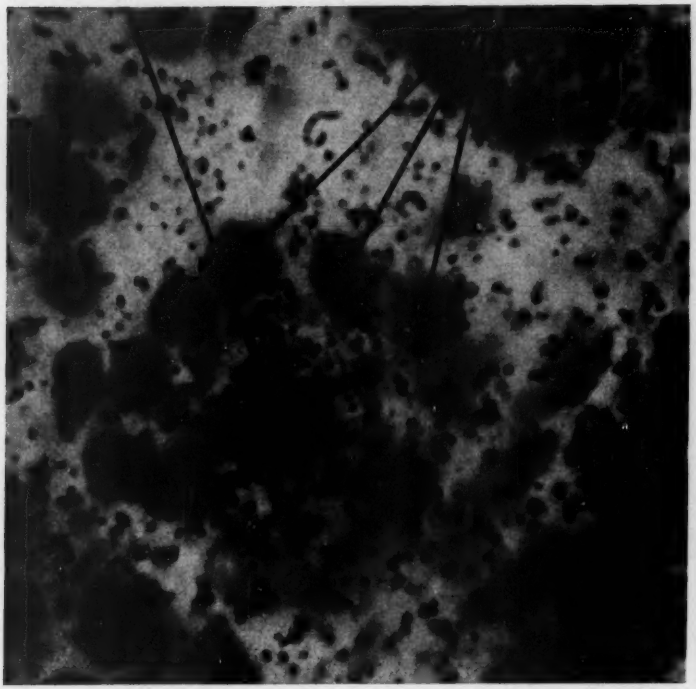

Fig. 10. Autoradiogram and a 5 μ section of mouse pancreas 60 minutes after intravenous injection of S^{35}-DL-methionine. The black spots indicate deposition of radioactivity. The highest activity is seen in the lumen of the acinus. The focal plane is between section and emulsion, making both cells and silver grains unsharp.
Eastman Kodak stripping film. Exposure time: 42 days. ×1100.

pattern. The activity was concentrated more in the centers of the acini (figure 10) and was fairly abundant in the excretory ducts. A zone characterized by an especially high amount of activity formed round the islands of Langerhans (figure 11). It was observed mainly in rats, being less distinct in mice and guinea pigs; and it followed injections both of cystine and of methionine. As a rule it had disappeared about six hours after the injection.

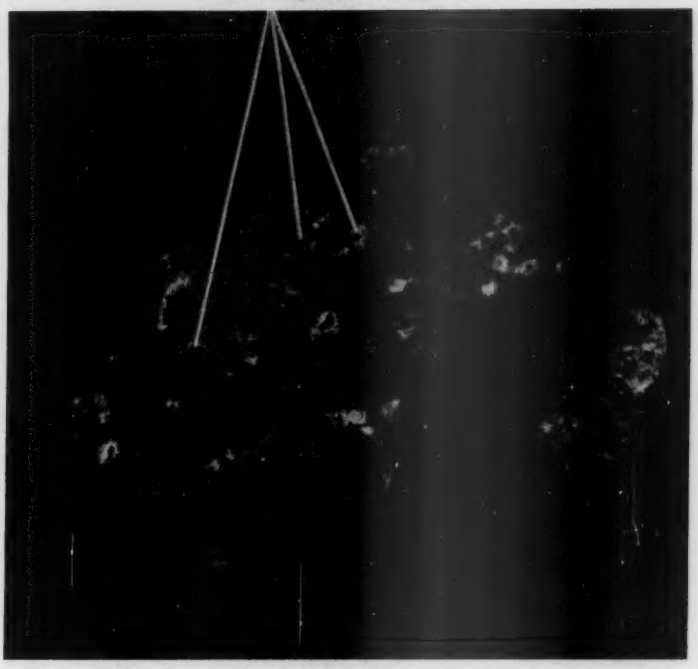

Fig. 11. Autoradiogram showing the distribution of radioactivity in rat pancreas 4 hours after intravenous injection of S^{35}-DL-methionine. White areas correspond to high radioactivity. The highest activity is seen in a zone around the islands of Langerhans.
Gaevert Dentus Rapid. Exposure time: 10 days. ×18.

Stripping film autoradiograms revealed that the peri-insular zone had a width equivalent to about two or three acini (figure 12). The cells in these acini showed a particularly high content of zymogen granules. The concentration of radioactive substance in different acini was relatively uniform at times shortly after the injection, but became more irregular after four hours.

One hour after the injection the radioactivity was concentrated largely to the centers of the acini. This pattern was even more

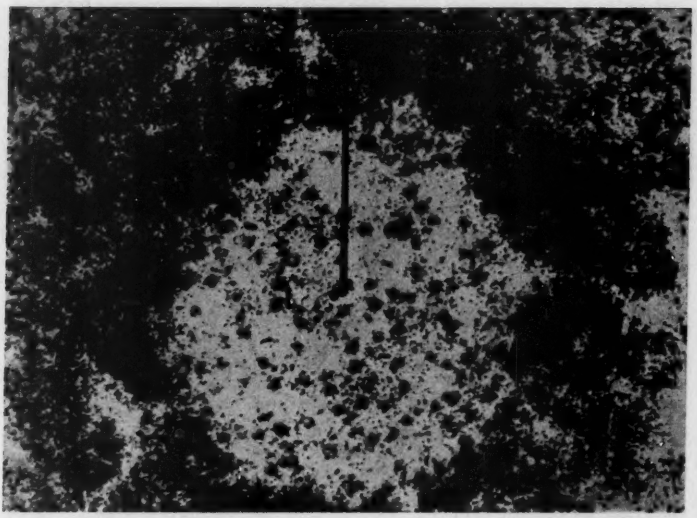

Fig. 12. Autoradiogram and a 5 μ section of rat pancreas showing the distribution of radioactivity 4 hours after intravenous injection of S^{35}-DL-cystine. The black spots indicate deposition of radioactivity. Note especially high activity in the exocrine tissue in a zone around the island of Langerhans but low activity in the island.
Eastman Kodak stripping film. Exposure time: 42 days. ×340.

pronounced after four hours. Since the border between the lumen of the acinus and the cell was indistinct, it was difficult to observe whether the radioactivity was localized in the zymogen granules or in the acinar lumina.

Autoradiograms of sections one hour after the injection showed a high content of radioactivity in the excretory ducts. Figure 13 illustrates an excretory duct and surrounding tissue four hours after the injection. The radioactivity was concentrated to the contents of the duct and was low in the latter's epithelium and the surrounding connective tissue.

The uptake of radioactivity in the islands of Langerhans was very low in comparison to that in the exocrine tissue during the

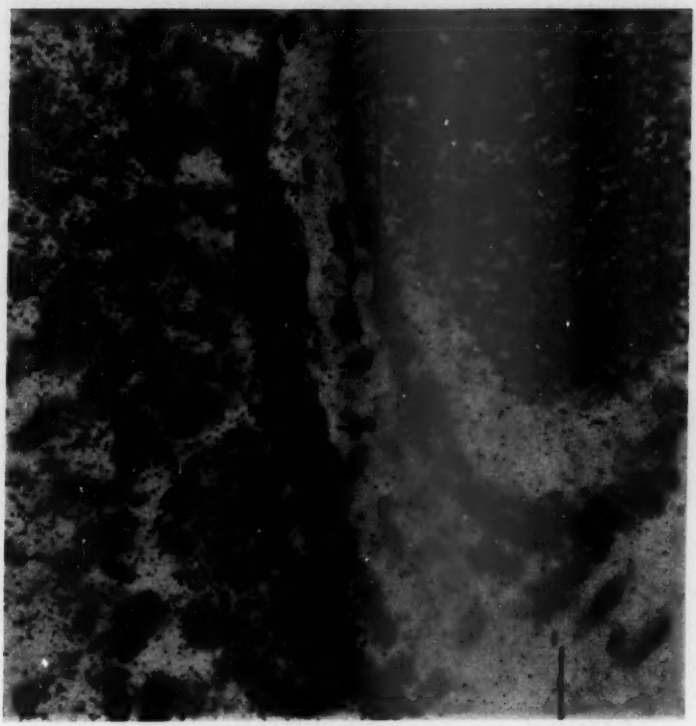

Fig. 13. Autoradiogram and a 5 μ section of mouse pancreas 4 hours after intravenous injection of S^{35}-DL-methionine. The black spots indicate deposition of radioactivity. Note high activity in the excretory duct and in the exocrine tissue but low activity in the island of Langerhans.
Eastman Kodak stripping film. Exposure time: 30 days. ×540.

first few hours, but 24 hours after the injection it was higher (figure 14). By that time, however, the activity in the exocrine tissue was lower than that in many of the other organs and tissues (figure 5).

Fig. 14. Autoradiogram showing the distribution of radioactivity in rat pancreas 24 hours after intravenous injection of S^{35}-DL-methionine. White areas correspond to high activity. Note high activity in the islands of Langerhans but low activity in the exocrine tissue.
Gaevert Dentus Rapid. Exposure time: 20 days. ×20.

DISCUSSION

From fifteen minutes to six hours after injection the distribution pattern for the amino acid uptake showed a conspicuous preponderance for the exocrine part of the pancreas by comparison with the islands of Langerhans. This also applied to an amino acid which constitutes a fairly high percentage of insulin; namely, cystine. The synthesis of insulin which is attributed to the *beta* cells of the islands, thus is not accompanied by anything

approaching the concentration of amino acids per producing cell as is the synthesis of pancreatic juice proteins.

The only explanation yet found for the marked concentration of radioactivity in the peri-insular zones lies in a slower discharge of the secretion therefrom than from the rest of the exocrine tissue. This observation on the peri-insular zones can be viewed in conjunction with SERGEYEVA's (1938) finding that zymogen granules are discharged more slowly therein. The cause of this delayed discharge is, however, obscure.

On stripping film autoradiograms the radioactivity in the acini shows first a diffuse localization in the cytoplasm, then increasing concentration in the acinar centers. The findings point to the following course of synthesis.

First an extremely rapid diffusion of the amino acids from the blood stream and their fairly even concentration in the cytoplasm; then, parallel with the formation of proteins in the cytoplasm, transport of the products to the acinar centers and their accumulation in zymogen granules, with occasional discharge of the latter.—The course observed autoradiographically was considerably faster than that reported by other authors (HIRSCH 1932, a, b and others) in studies on the resynthesis of zymogen granules following discharge with pilocarpine. The most plausible explanation of this is that the effect of a pilocarpine injection persists for several hours (page 16), so that the discharge of newly formed granules proceeds long after the injection, and normalization of the zymogen granule content of the exocrine cells is therefore delayed.

CHAPTER V

Occurrence of Radioactivity in the Protein Fraction and in the Trichloroacetic Acid Soluble Fraction of Pancreas after Intravenous Injection of S^{35}-DL-Methionine

The autoradiographic investigations in Chapter III have shown that the pancreas has an extremely rapid uptake of intravenously injected labelled amino acids. The radioactivity has, only five minutes after the injection, a high concentration in the pancreas in relation to other organs, and reaches its maximum about 30 minutes after the injection. It then falls and, approximately six hours after the injection, is very slightly higher in the pancreas than in other organs. This variation in radioactivity has been considered chiefly to demonstrate the incorporation of the labelled amino acids into the digestive enzyme proteins formed by the pancreas, as well as the excretion of those proteins. DALY and MIRSKY (1952) elucidated the rate at which certain enzymes were resynthesized in mouse pancreas after the enzymes had been discharged by stimulation of the secretion with pilocarpine. They found that although the enzyme content fell after discharge of the enzymes, the total protein content was virtually unchanged. In their opinion, elimination of the enzyme proteins is followed by a swift resynthesis of precursor protein, which is then transformed into the various enzyme proteins. FARBER and SIDRANSKY (1956) investigated, similarly, the secretion and resynthesis of enzymes in rat pancreas after treatment with cholinergic agents, and found that the total protein content decreased after stimulation. Contrary to DALY and MIRSKY they considered that the excretory enzymes of the pancreas were synthesized directly from free amino acids, without any intermediate substances.

ALLFREY, DALY and MIRSKY (1953), DALY, ALLFREY and MIRSKY (1955), and FARBER and SIDRANSKY (1956) used labelled amino acids for studying the synthesis of protein in the pancreas.

They measured the incorporation of amino acids *in vivo* and found a high uptake by the proteins of the pancreas. No data, however, are available on the rate of synthesis, and these examinations concerning the incorporation of labelled amino acids into pancreatic proteins have generally been made a relatively long time after injection of those acids. As a rule, the earliest observations have been recorded 30–90 minutes after intraperitoneal injection of the amino acids. HULTIN (1950) and BORSOOK *et al.* (1950) among others, have shown that the synthesis of protein in the liver is extremely rapid; a high incorporation into the proteins occurs only a minute or so after injection of labelled amino acids.

This chapter is concerned with the uptake of radioactivity in the pancreas of mice and guinea pigs in the first few hours after the injection of S^{35}-DL-methionine. The uptake was compared in normally fed mice, in mice and guinea pigs starved for 24 hours, and in mice and guinea pigs starved for 24 hours and then treated with pilocarpine. The mice received an injection of 0.1 μC S^{35}-DL-methionine per gram of body weight and the guinea pigs 0.05 μC per gram of body weight. Pilocarpine-treated mice and guinea pigs received an injection of 50 and 15 μg pilocarpine hydrochloride per gram of body weight respectively, 90 minutes before and coincident with the injection of S^{35}-methionine. With mice the injections were made in a tail vein; with guinea pigs, in a cubital vein. The animals were sacrificed 5, 10, 20, 40, 80 and 160 minutes after the injection, after which the pancreas was removed and homogenized. The proteins were precipitated with TCA and the TCA-soluble and the protein-bound radioactivity were then determined.

RESULTS

In the protein fraction of normally fed mice the radioactivity per milligram of protein was already high in the sample taken five minutes after the injection (figure 15), and it rose to a maximum after 80 minutes. From 40 to 160 minutes after the injection there was only a slight difference. In the individual experiments the

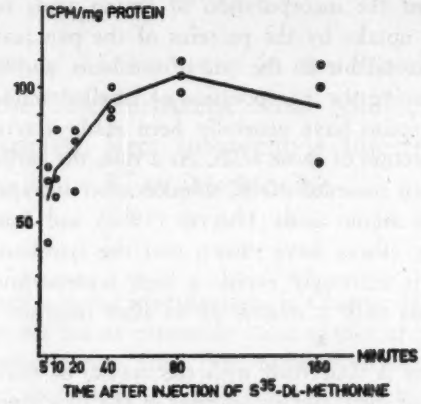

Fig. 15. Variation with time in the radioactivity of the protein fraction of mouse pancreas after intravenous injection of S^{35}-DL-methionine in normally fed mice. Each circle indicates the value for combined samples from two animals.

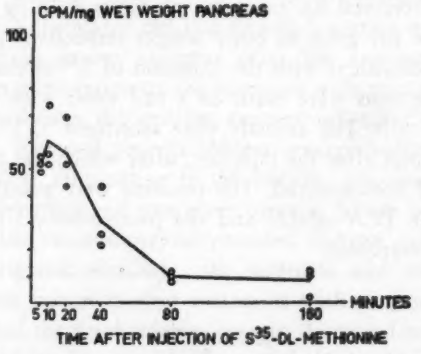

Fig. 16. Variation with time in the radioactivity of the trichloroacetic acid soluble fraction of mouse pancreas after intravenous injection of S^{35}-DL-methionine in normally fed mice. Each circle indicates the value for combined samples from two animals.

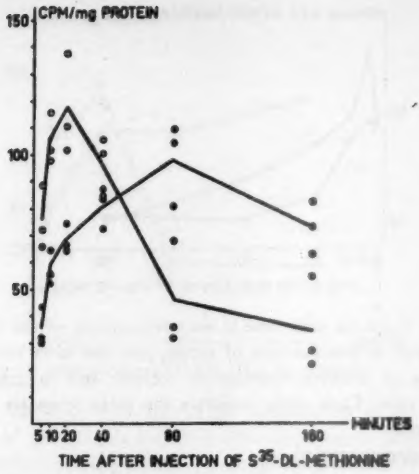

Fig. 17. Variation with time in the radioactivity of the protein fraction of mouse pancreas after intravenous injection of S^{35}-DL-methionine in starved and in pilocarpine-treated mice. Each circle indicates the value for combined samples from two animals. The lines represent mean values.

● starved
○ pilocarpine-treated

results differed considerably for determinations 5, 10, 20 and 160 minutes after the injection, but the range was very small for those made after 40 and 80 minutes. The findings indicate that S^{35}-methionine taken up by the pancreas is incorporated into protein very swiftly; after only five minutes the radioactivity in the protein fraction amounted to about 60 per cent of its maximum 80 minutes after the injection. The rise occurring between 40 and 80 minutes was not more than about 10 per cent, showing that almost the maximum was reached in the protein fraction after about 40 minutes.

The radioactivity in the TCA-soluble fraction (figure 16) was high five minutes after the injection and attained its maximum after 10 minutes. It then fell rapidly, though was still appreciable after 160 minutes.

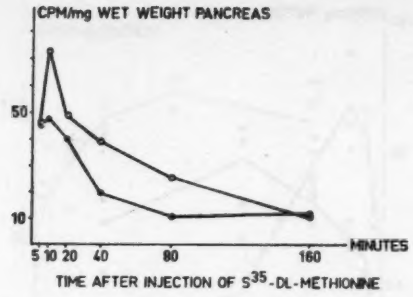

Fig. 18. Variation with time in the radioactivity of the trichloroacetic acid soluble fraction of mouse pancreas after intravenous injection of S^{35}-DL-methionine in starved and in pilocarpine-treated mice. Each circle indicates the mean from six animals.
● starved
○ pilocarpine-treated

The incorporation of S^{35}-methionine into the protein fraction in starved, pilocarpine-treated mice differed from that in normally fed mice and those which had been starved but not treated with pilocarpine (figure 17). During the first 40 minutes after injection of methionine the radioactivity was substantially greater in pilocarpine-treated than in untreated mice. In the former animals the maximum radioactivity in the protein fraction was found after 20 minutes; in the latter, after 80 minutes. A decrease was observed between 20 and 40 minutes after injection in the pilocarpine group, but the fall was especially pronounced after 40–180 minutes, when it amounted to about 50 per cent. During the corresponding period there was a rise in the untreated animals. The radioactivity in the TCA-soluble fraction (figure 18) was higher in pilocarpine-treated than in untreated animals. In both groups the highest content of radioactivity was found in the samples taken 10 minutes after the injection.

The incorporation of S^{35}-methionine into the pancreatic protein fraction was compared in starved guinea pigs, with and without pilocarpine treatment (figure 19). During the first 40 minutes after the injection the radioactivity was higher in the treated

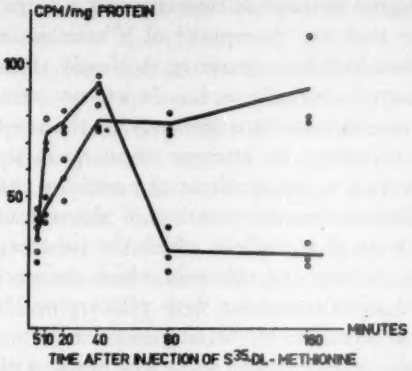

Fig. 19. Variation with time in the radioactivity of the protein fraction of guinea pig pancreas after intravenous injection of S^{35}-DL-methionine in starved and in pilocarpine-treated guinea pigs. Each circle indicates the value from one animal. The lines represent mean values.

● starved

○ pilocarpine-treated

animals, but in both groups it rose. In the pilocarpine group it showed, between 40 and 80 minutes, a decrease of about 75 per cent, suggesting that there was a heavy secretion of labelled proteins. In the untreated group the radioactivity increased throughout the period of observation but the rise was very slight between 40 and 160 minutes.

DISCUSSION

The incorporation of S^{35}-DL-methionine into the pancreatic proteins was compared in normally fed animals, in others starved for 24 hours, and in animals starved for 24 hours and then treated with pilocarpine. In the starved animals the secretory activity was not appreciably reduced by comparison with normally fed animals (table I); but it was assumed that by using animals starved for 24 hours the individual variation in pancreatic secretion would

be in some degree eliminated, thus ensuring a more homogeneous material. For studying the uptake of S^{35}-methionine in animals whose pancreas had been secreting profusely, mice and guinea pigs were injected with pilocarpine. It was not ascertained if the dose always caused maximum discharge of the enzymes. In these experiments, therefore, no attempts were made to compare extreme conditions, i. e., the synthesis of protein at maximum secretion in relation to that on cessation of the secretion. The only comparisons were of animals in which the pancreas showed normal secretory activity and others in which the secretory activity was increased after treatment with pilocarpine. Stimulation of the pancreatic secretion by feeding starved animals, yields far more variable results than does treatment with pilocarpine (DALY and MIRSKY 1952).

The observed values for the pancreatic content of amylase suggest that in mice and guinea pigs the pancreas has an undiminished secretory activity after 24 hours' starvation, since the difference between normally fed animals and those starved for 24 hours was minimal (table I). This probably is not due to a suppressed or depressed synthesis of protein, for no difference in the uptake of radioactivity in the protein fraction was observed between normally fed animals and those starved for 24 hours (figures 15 and 17).

The autoradiographic investigations reported in Chapter III showed that the pancreas had a rapid and high uptake of intravenously injected amino acids by comparison with other organs. Almost maximum uptake was found only 5 minutes after the injection (figure 4). It was evident from the results that the radioactivity in the free amino acid fraction reached its maximum after only 10 minutes, then gradually fell (figure 16). This behavior was observed both in normally fed animals and in starved and pilocarpine-treated animals. In starved and in normally fed mice the specific radioactivity in the protein fraction rose during the first 80 minutes after the injection, and subsequently decreased. The autoradiographic findings demonstrated, however, that the total uptake of radioactivity in the pancreas reached its maximum about 30 minutes after the injection, and that some of the activity disappeared between 30 and 60 minutes after the injection

(figure 4). It is thus evident that secretion of formed protein products may occur even as early as that.

In the present investigation the incorporation of radioactivity into the proteins was expressed as radioactivity per milligram of protein.—Non-radioactive protein dilutes radioactive protein to a degree depending on the amount of digestive enzyme protein stored in the cell. The structural proteins may be assumed to remain fairly constant under the relevant conditions, and hence will not appreciably affect the results obtained. In mice the enzyme proteins constitute about 20 per cent of the dry weight (DALY and MIRSKY 1952), and a substantial part of them may be discharged after injection of pilocarpine. The radioactivity in the protein fraction rose after pilocarpine treatment; this may point to an increased synthesis of protein, as was assumed by ALLFREY, DALY and MIRSKY (1953) and FARBER and SIDRANSKY (1956), though no definite conclusion can be drawn from the present results in respect to the isotope dilution effect.

The rapid decrease of radioactivity in the proteins in the pilocarpine-treated mice and guinea pigs suggested that a secretion of protein products, into which the injected amino acid had been incorporated, occurred between 40 and 80 minutes after the injection. The decrease of radioactivity following pilocarpine treatment, may be attributed almost entirely to secretion of enzyme proteins (figure 19), since there had scarcely been time for fresh accumulation of inactive enzyme proteins, leading to isotope dilution.

CHAPTER VI

Incorporation of S^{35}-DL-Methionine into the Proteins of Various Cell Fractions of the Pancreas

Differential centrifugation in sucrose solution makes it possible to separate the cell components, nuclei, mitochondria and microsomes (HOGEBOOM, SCHNEIDER and PALADE 1948) as well as zymogen granules from the pancreas (HOKIN 1955).

The method has opened up possibilities of elucidating those cell structures into which labelled substances are incorporated, or those in which certain substances such as enzymes are localized.

The investigations of HULTIN (1950), BORSOOK *et al.* (1950) and KELLER, ZAMECNIK and LOFTFIELD (1954), among others, have shown that the proteins in the liver microsomal fraction have the highest initial specific activity after injection of labelled amino acids. The same has proved to be true of the pancreas (ALLFREY, DALY and MIRSKY 1953; SIEKEVITZ and PALADE 1958 b). This suggests that the bulk of the proteins or their precursors formed in the cells are produced in the microsomal fraction, then transported to other parts of the cells.

The pancreas shows a peculiar behavior insofar as the enzyme proteins are stored in the zymogen granules before being discharged. Ever since the discovery of zymogen granules last century, those cellular elements have been regarded as storage sites for the excretory enzymes. This observation is based on the storage and discharge of the zymogen granules with starvation and feeding respectively, and on the fact that those granules decrease after enzymes have been eliminated by administration of pilocarpine or other cholinergic drugs. Fluorescine-labelled antibodies specific for some pancreatic enzymes may be localized in the zymogen granules (MARSHALL 1954). By means of differential centrifugation of pancreas homogenate from dogs, HOKIN (1955) was able to isolate a fraction which contained chiefly zymogen granules; and he showed that it had a higher

amount of proteolytic activity, amylase and lipase compared with the whole homogenate. These results were recently verified by LAIRD and BARTON (1957, 1958) in rat pancreas and by SIEKEVITZ and PALADE (1958 a) in guinea pig pancreas. They found that amylase and proteolytic activity, respectively, were most abundant in the zymogen granule fraction.

The aim of the investigations reported in this chapter was to study the incorporation of radioactivity into the proteins of various cell fractions of guinea pig pancreas at varying intervals after the injection of S^{35}-DL-methionine, with special reference to the uptake of radioactivity by the zymogen granules.

The incorporation was studied in normally fed guinea pigs, in others starved for 24 hours, and in animals starved for 24 hours and then treated with pilocarpine. The animals were injected with 0.05 μC S^{35}-DL-methionine per gram of body weight. Pilocarpine-treated guinea pigs received an injection of 15 μg pilocarpine hydrochloride per gram of body weight 90 minutes before and coincident with the injection of methionine. All injections were given intravenously into a cubital vein. The guinea pigs were sacrificed 10, 20, 40, 80 and 160 minutes after the injection of methionine. The pancreas was removed and the various cell fractions separated as described on page 25.

RESULTS

In normally fed guinea pigs the incorporation of S^{35}-methionine was highest in the protein of the microsomal fraction by comparison with other cell fractions during the first 40 minutes after the injection (figure 20). In the first-named fraction it rose almost linearly up to its maximum 40 minutes after the injection. It fell considerably between 40 and 80 minutes, and also decreased 80–160 minutes, after the injection.

In the mitochondrial fraction the radioactivity per milligram of protein increased greatly from 10 to 20 minutes after the injection but then showed only minor changes. The radioactivity in the nuclear and supernatant fractions exhibited very small

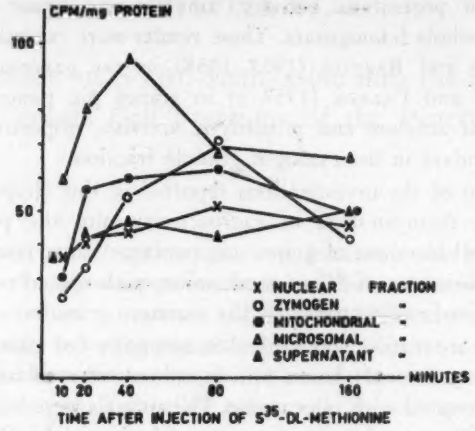

Fig. 20. Variation with time in the radioactivity of the proteins of various cell fractions of guinea pig pancreas after intravenous injection of S^{35}-DL-methionine in normally fed guinea pigs. Each value indicates the mean from two animals.

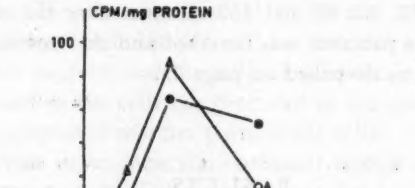

Fig. 21. Variation with time in the radioactivity of the proteins of various cell fractions of guinea pig pancreas after intravenous injection of S^{35}-DL-methionine in starved guinea pigs. Each value indicates the mean from two animals.

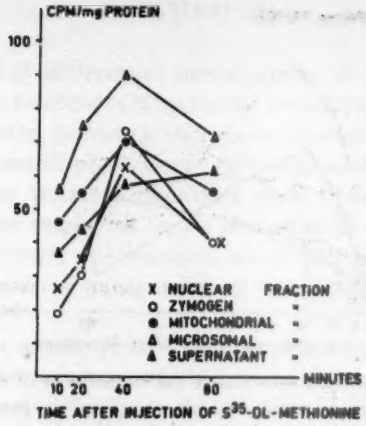

Fig. 22. Variation with time in the radioactivity of the proteins of various cell fractions of guinea pig pancreas after intravenous injection of S^{35}-DL-methionine in pilocarpine-treated guinea pigs. Each value indicates the mean from two animals.

variations and had already reached about 80 per cent of its maximum after 10 minutes.

The corresponding activity in zymogen granules rose almost linearly during the first 80 minutes, when it had attained its maximum. It was lower than in the other fractions for the first 20 minutes, but from then up to 80 minutes it increased substantially, coincident with a fall in the microsomal fraction.

Starved guinea pigs showed great similarities to normally fed with regard to the incorporation of methionine (figure 21). The radioactivity was higher in the microsomal fraction than in any of the others and reached its maximum 40 minutes after the injection. This was the case even in pilocarpine-treated animals (figure 22). As regards the radioactivity in the zymogen granules there were major differences between pilocarpine-treated and untreated animals. In the latter, as in normally fed animals, the activity rose throughout the period of observation, but in the former it decreased substantially between 40 and 80 minutes after the injection of S^{35}-methionine.

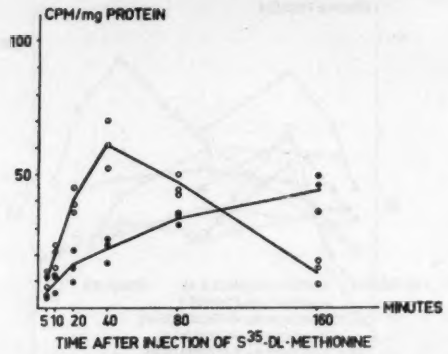

Fig. 23. Variation with time in the radioactivity of the proteins of the zymogen granule fraction of guinea pig pancreas after intravenous injection of S^{35}-DL-methionine in starved and in pilocarpine-treated guinea pigs. Each circle indicates the value for combined samples from two animals. The lines represent mean values.

● starved

○ pilocarpine-treated

In other cell fractions the radioactivity showed its maximum 40 minutes after the injection, and here there was little difference between pilocarpine-treated and untreated animals.

Figure 23 depicts an experiment in which the radioactivity in the proteins of the zymogen fraction was compared in the two groups. This activity showed a fairly linear rise in both groups for the first 40 minutes after the injections. However, the curves diverged by virtue of a much swifter rise in the pilocarpine group. The starved guinea pigs showed a continuous increase of the radioactivity in zymogen granules throughout the relevant period, but the pilocarpine-treated animals a decrease 40–160 minutes after the injection.

Forty minutes after the injection the radioactivity in the zymogen fraction of the pilocarpine group was approximately double that in the untreated group, but after 160 minutes the reverse was the case.

DISCUSSION

The method of differential centrifugation of homogenate for obtaining cell fractions (HOGEBOOM, SCHNEIDER and PALADE 1948) has assumed increasing importance in studies on the function and biochemical properties of different cellular components. It is chiefly for isolating subcellular parts from liver that the method has been employed. In the present work it was used for separating pancreatic cell fractions, which may be considered to have had a degree of purity sufficing for investigation of their characteristics.

Here, certain sources of error must be borne in mind. For instance, the pancreas is not composed solely of exocrine tissue but is rich in connective tissue, vessels and islet tissue. However, this source of error is insignificant, for the enzyme content and radioactivity (figure 9) are low in those structures and thus influence the results almost solely by virtue of dilution. Another source of error is the fact that ruptures of cell components occur. With cell ruptures there will be disintegration of mitochondria, nuclei and zymogen granules (HOGEBOOM and SCHNEIDER 1955). The results nevertheless show that division into cell fractions is readily accomplished in guinea pig pancreas, and that the zymogen granules can be isolated *ad modum* HOKIN (1955).

In this investigation only the radioactivity in the total proteins of different cell fractions was studied. It would have been advantageous, of course, to isolate the specific enzyme proteins and to determine the radioactivity therein; but the results suggest that the radioactivity in the total proteins, and notably the zymogen granules, reflects the synthesis of the enzyme proteins. True, it must be assumed that a part is incorporated into cell structures, but presumably that part is low in relation to the proportion incorporated into the secretory products, according to the results reported in Chapters III and IV.

During the first of the periods observed, the radioactivity was higher in the proteins of the microsomal than in any other fraction (figure 20). This finding agrees well with the reported incorporation of labelled amino acids into liver proteins (HULTIN 1950; BORSOOK *et al.* 1950). The activity in the microsomal frac-

tion was already high in the samples taken 10 minutes after the injection, then rose slightly and reached its maximum after 40 minutes. On the whole, the other cell fractions showed a similar course, aside from the activity in zymogen granules.

These results accord on the whole with those obtained by SIEKEVITZ and PALADE (1958 b), who used C^{14}-DL-leucine. However, they found the maximum radioactivity in the microsomal fraction only 15 minutes after the injection, and a higher relative activity in the zymogen granule fraction. This discrepancy is probably due to variations in the experimental conditions: SIEKEVITZ and PALADE used guinea pigs that had been starved for 48 hours, as well as a different cell fractionation technique.

The present results concerning the incorporation of S^{35}-methionine into the different cell fractions may be considered to show both the formation of new proteins and the transport of newly formed proteins from one cell fraction to another. The levelling out of a curve for radioactivity in the proteins of some cell fraction may be assumed to demonstrate a steady state of the two processes.

The radioactivity in the microsomal fraction was already high 10 minutes after the injection, but then did not rise so swiftly as that in other fractions and in fact decreased 40 minutes after the injection. This may have been due to a release of formed proteins for building up zymogen granules. There are numerous theories as to how this process takes place and in which part of the cell. A number of authors have assumed that the Golgi apparatus may serve as a condensing membrane, which concentrates the products synthesized in other parts of the cell into granules, which are then excreted (SJÖSTRAND and HANZON 1954).

Possibly other cellular components than the microsomes possess the ability to incorporate amino acids into protein. It has been thought that the nuclear and mitochondrial fractions may synthesize protein (ALLFREY, MIRSKY and OSAWA 1957; BATES, CRADDOCK and SIMPSON 1958), though there is no conclusive evidence of this. In the present study incorporation of radioactivity into other cell fractions than the microsomes was observed. Whether this was due to synthesis of protein in those fractions or to uptake of formed protein from the microsomal fraction

was not clear. The separated fractions, as mentioned earlier, were not cytologically homogeneous; evaluation was rendered difficult by contamination with microsomal material in the fractions.

Starved and pilocarpine-treated animals showed a conspicuous difference with regard to radioactivity in the zymogen granules; for that activity fell substantially 40 minutes after injection in the latter group, but rose in the former. This suggests that the bulk of newly formed enzyme proteins is already localized in the zymogen granules 40 to 80 minutes after the injection and that secretion of enzyme protein may already occur at that time.

Guinea pigs in which the pancreatic secretion had been stimulated with pilocarpine showed a higher radioactivity in the zymogen granule fraction than did the starved animals. This was probably due largely to an effect of isotope dilution and not to increased synthesis of protein, for in pilocarpine-treated animals the zymogen granule fraction obtained by centrifugation was only about one half of that found in starved animals (table IV). On the other hand, there was no major difference between the two groups with regard to the amount of radioactivity in other cell fractions.

It is evident from the present results that the synthesis of enzyme proteins and their accumulation in the zymogen granules proceed far more swiftly than indicated by the cytologic investigations of HEIDENHAIN (1875), HIRSCH (1932 a, b), and others. These results are more consistent with the observations of KOMAROV, LANGSTROTH and MCRAE (1939) and LANGSTROTH, MCRAE and KOMAROV (1939), who calculated with a resynthesis time of 2–3 hours after discharge of the enzymes with secretin.

In the present investigations pilocarpine was used in elucidation of the rate at which the newly produced enzymes were ready for excretion; for by thus stimulating the pancreas the enzymes were eliminated more rapidly.—It may be assumed from figure 23 that the synthesis rate does not differ appreciably in starved and in pilocarpine-treated animals, but in the latter group discharge of the formed zymogen granules is accelerated, with a consequent fall in radioactivity.

The findings show that synthesis of the enzyme proteins and their accumulation in the zymogen granules constitute a very

swift process which, in the animals studied here, might occur within one hour after the precursors— the amino acids—have been taken up by the cells. The uptake of amino acids, which occupies only a few minutes (figure 2), and their incorporation into proteins proceed rapidly, and the longest time is evidently taken for the newly formed proteins to concentrate in the zymogen granules before they can be excreted.

CHAPTER VII

Occurrence of Radioactivity in the Pancreatic Juice Proteins Following Intravenous Injection of Labelled Amino Acids

Several investigations have shown that certain substances appear in the pancreatic juice very shortly after intravenous injection. BALL (1930) demonstrated that sodium and potassium were present in that juice immediately after they had been injected intravenously in concentrated solution. MONTGOMERY, SHELINE and CHAIKOFF (1941) observed radioactivity in the juice within a few minutes after intravenous injection of radioactive sodium, as did BALL *et al.* (1941) in the case of C^{14}-labelled bicarbonate. JUNQUEIRA, HIRSCH and ROTSCHILD (1955) and ROTSCHILD, HIRSCH and JUNQUEIRA (1957) determined the time of appearance of radioactive proteins in the pancreatic juice of rats following injection of labelled amino acids. They found no appreciable radioactivity until 50 minutes after the injection, and the maximum was reached after 2–5 hours. The time of this peak varied for different amino acids; for C^{14}-glycine, for instance, it was $2^3/_4$ hours, and for C^{14}-histidine about 4 hours, after the injection.

In this section it was sought to elucidate the interval between intravenous injection of labelled amino acids in cats and the first appearance of radioactivity in the pancreatic juice. In order to determine the rate of synthesis, it was desirable to obtain the newly produced enzyme proteins in the pancreatic juice as soon as they were in an excretable form. For this purpose the pancreas was stimulated to ensure a continuous secretion.

EXPERIMENTAL

OPERATION

The cats were anesthetized with chloralose and urethane (intramuscularly 6 ml per kilogram of body weight of a solution

containing 1.1 g chloralose and 3.6 g urethane per 100 ml). They were tracheotomized and a cannula was inserted into the femoral vein. The abdomen was opened in the mid-line. The pancreatic juice was collected by introducing a polyethylene tube (Intramedic, Clay Adams PE 50) into the main pancreatic duct close to its junction with the duodenal papilla.

SECRETION OF PANCREATIC JUICE

The cat has a hunger secretion which may continue for about 24 hours (WILANDER and ÅGREN 1932), though it is slight. In the present investigations it was desirable to obtain the newly produced enzymes in the pancreatic juice as soon as possible, and hence stimulants were used to ensure a continuous flow. The secretion was stimulated with secretin and pancreozymin, which preparations were kindly supplied by Professor E. Jorpes, Karolinska Institutet. Secretin stimulates the fluid and bicarbonate secretion (MELLENBY 1925, and others). Pancreozymin acts on the enzyme secretion in the same way as pilocarpine and other cholinergic drugs (HARPER and RAPER 1943). The secretin and pancreozymin used in these experiments were substances prepared *ad modum* JORPES and MUTT (1953 a, b, 1955 a, b). The secretin was a highly purified substance containing approximately 10,000 cat units per milligram (WILANDER and ÅGREN 1932), while the pancreozymin was a more crude preparation. Each of the stimulants was dissolved in physiologic saline and injected continuously at the rate of about 10 ml per hour, corresponding to a dose of some 50 cat units secretin and 2 mg pancreozymin per kilogram of body weight per hour. The infusion rate was kept constant by using an infusion apparatus.

COLLECTION OF PANCREATIC JUICE

The pancreatic juice was collected in test tubes placed in iced baths and changed every 5 minutes the first hour after the injection and then every 15 minutes. Samples of the juice were frozen and stored at —20° C until the chemical and radiometric determinations were carried out.

RESULTS

APPEARANCE OF RADIOACTIVITY IN THE PANCREATIC JUICE

Secretion of pancreatic juice was obtained almost immediately after injection of pancreozymin and secretin, and the flow remained fairly constant throughout the experiment. In a few animals, however, the volume of pancreatic juice decreased after an hour or so. A larger amount of the stimulants was then injected and the volume returned to its former level. The volume obtained was approximately 5–6 ml per hour, and the protein content was about 1–2 g per 100 ml. This content was higher during the first few minutes after stimulation than it was later on. Figure 24 shows a typical experiment in which the volume of pancreatic juice and its amylase and protein contents were studied during continuous injection of pancreozymin and secretin in the dosage used in these studies. It will be seen from the figure that the protein content fell from just over 2 g per 100 ml to about 1 g per 100 ml after one hour, but then remained at that concentration throughout the rest of the experiment. A greater volume could be obtained by increasing the concentration of secretin in the infusion solution, but the juice then had a lower protein content. In a few experiments it was sought to raise the protein content by injecting a larger dose of pancreozymin, but the effect was not appreciable.

In this section it was desirable to have pancreatic juice with a constant protein content during the whole of the experiment. This seems to have been achieved in the majority of experiments, as of about one hour from the start of the injection of pancreozymin and secretin. The protein content was then approximately 1 g per 100 ml and fell only very slightly during the 5–10 hours which the various experiments lasted. As a rule the radioactive amino acid was injected 30–45 minutes after stimulation with secretin and pancreozymin had begun, by which time the protein content was fairly constant.

In the majority of experiments S^{35}-DL-methionine was injected; only in a few were other labelled amino acids used. The

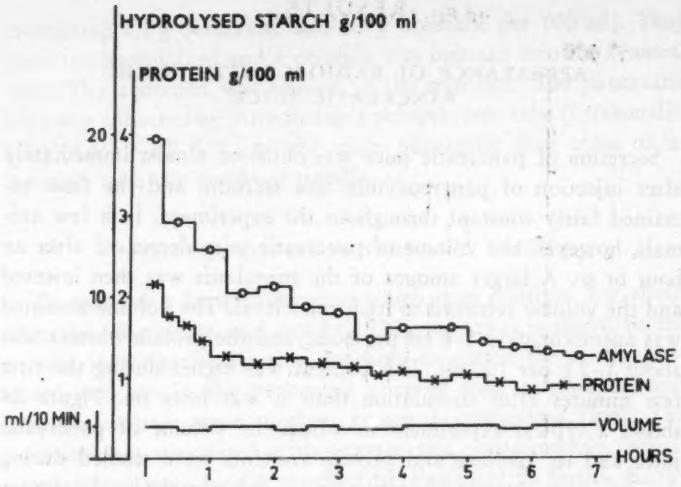

Fig. 24. Variation with time in volume, protein content and amylase content of cat pancreatic juice following stimulation of the secretion by continuous infusion of secretin and pancreozymin. The pancreatic juice was collected every 15 minutes.

results obtained with methionine will therefore be reported in detail; those associated with other amino acids will merely be cited for comparative purposes. The protein content and radioactivity were determined in all samples. As a rule every third sample was assayed for amylase. In the figures the radioactivity in the pancreatic juice is expressed as CPM/ml (counts per minute /ml) or as CPM/mg protein. The amount of amino acids injected is given in microcuries per kilogram of body weight.

Figure 25 shows the occurrence of radioactivity in pancreatic juice after injection of 10 μC S^{35}-DL-methionine per kilogram of body weight. There was a high content of radioactivity only five minutes after the injection, but the radioactivity then fell and, after 15–30 minutes, was insignificant. This course was observed in all experiments, though the concentration of radioactivity varied during the first few minutes after the injection. The radioactivity obtained in the first 15 minutes was almost wholly TCA

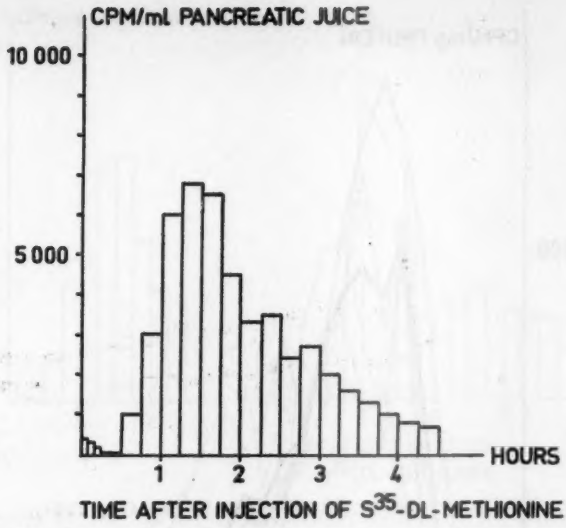

Fig. 25. Appearance of radioactivity in cat pancreatic juice after intravenous injection of S^{35}-DL-methionine. The secretion was stimulated by continuous infusion of secretin and pancreozymin. The juice was collected every 5 minutes for the first hour after the injection, and then every 15 minutes.

soluble (page 77). From 30 to 45 minutes after the injection the radioactivity increased and reached its maximum 1–2 hours after the injection. The radioactivity found after 30 minutes and later was almost entirely protein-bound (page 78). The time of its maximum varied in the different experiments. Figure 26 shows six experiments in which 10 μC S^{35}-DL-methionine per kilogram of body weight was injected. The mean curve indicates a maximum about 1–2 hours, and a fall in radioactivity usually between 2 and 3 hours, after the injection. In a few instances there is a distinct radioactivity peak approximately two hours after the injection, but in others no peak is evident. In some cases the radioactivity fell rapidly in the pancreatic juice and was negligible 4–5 hours after the injection.

In these experiments the activity was expressed as CPM/mg protein. The results showed that the decrease of radioactivity

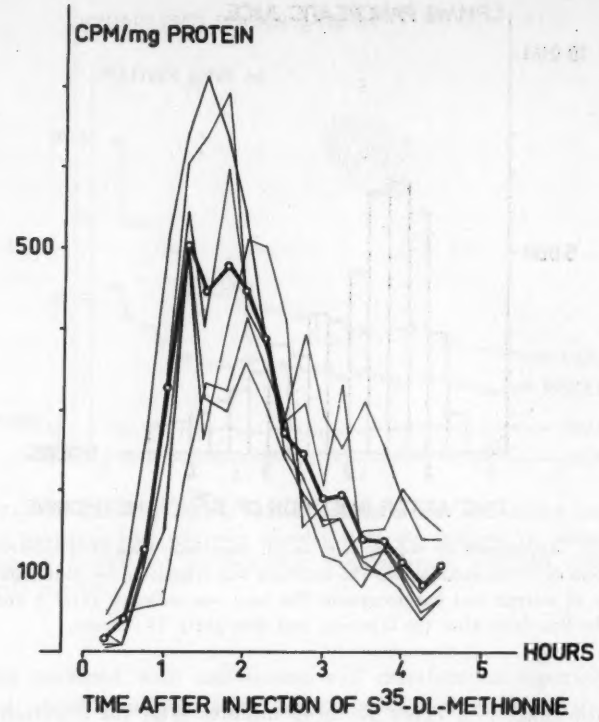

Fig. 26. Variation with time in the specific activity of the proteins of cat pancreatic juice following intravenous injection of S^{35}-DL-methionine. The figure shows the results from six cats. Experimental conditions the same as in fig. 25.

— single experiment

▬ mean curve

about 3–4 hours after the injection was due, not to a reduction in the amount of protein during the experiment but to a lower S^{35} content of the proteins. In order to study this further and to ascertain if the pancreatic ability to synthesize proteins diminished with prolonged secretion, a second injection of S^{35}-DL-methionine was given, in a few experiments, about four hours after the first. The pancreatic juice showed two distinct radio-

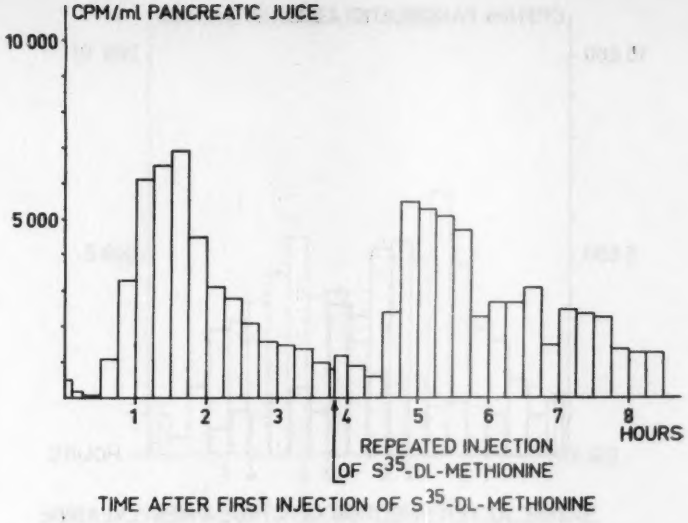

Fig. 27. Appearance of radioactivity in cat pancreatic juice after two intravenous injections of S^{35}-DL-methionine. The injections were given 230 minutes apart. Experimental conditions the same as in fig. 25.

activity peaks, occurring 1–2 hours after each injection (figure 27). These peaks were almost identical in size and form. The protein content remained at about 1 g per 100 milliliters throughout the experiment. Radioactivity in the juice was appreciable only a few minutes after the first injection but then decreased and, after 15–30 minutes, had disappeared. It showed an elevation in the sample taken five minutes after the second injection; then fell and was lowest after 25–40 minutes. Subsequently it rose again and reached its maximum 1–2 hours after the second injection.

The protein content remained at about 1 g per 100 ml throughout the experiment. These findings show that the radioactivity peak observed about 1–2 hours, and the fall about 3–4 hours, after the injection were not due to fluctuations in the protein content. Nor did the power of synthesis appear to be appreciably affected by prolonged secretion, for the concentration of radio-

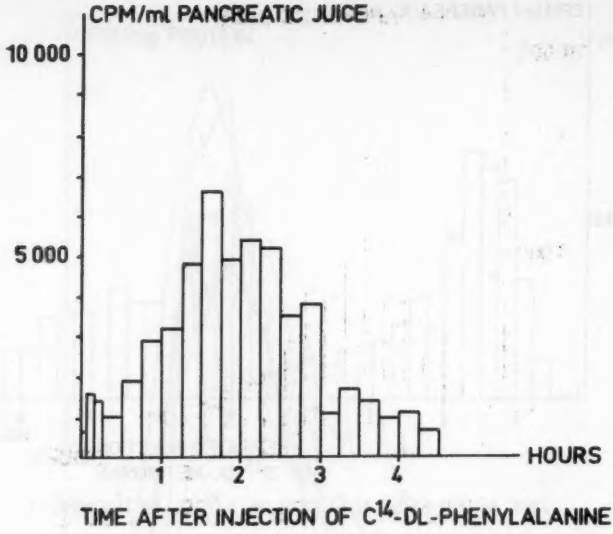

Fig. 28. Appearance of radioactivity in cat pancreatic juice after intravenous injection of C^{14}-DL-phenylalanine. Experimental conditions the same as in fig. 25.

activity in the pancreatic juice was approximately the same after the two injections.

In a few experiments the percentual excretion of injected S^{35}-DL-methionine was determined during the first four hours after the injection. During that period about 3–4 per cent of the amount injected was discharged in the pancreatic juice.

Figures 28 and 29 show the results after injection of C^{14}-DL-phenylalanine and S^{35}-DL-cystine. The radioactive content of the pancreatic juice following injection of those amino acids exhibited a pattern very similar to that after injection of S^{35}-DL-methionine. No conspicuous difference was found.

In one experiment the high dose of 100 μC S^{35}-DL-methionine per kilogram of body weight was injected. High activity was found in the pancreatic juice the first few minutes, and the activity then fell to a low value 15–30 minutes after the injec-

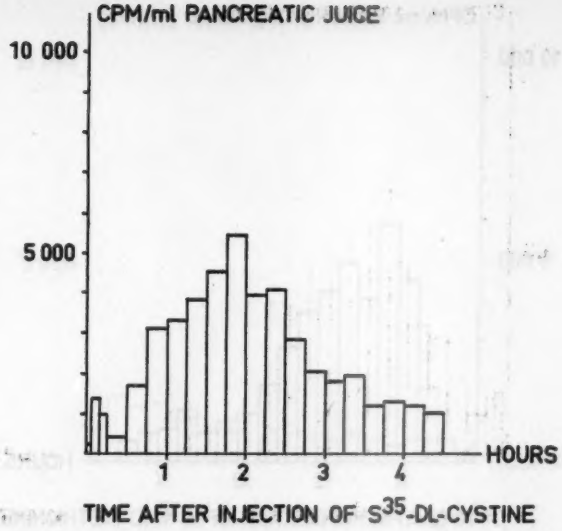

Fig. 29. Appearance of radioactivity in cat pancreatic juice after intravenous injection of S^{35}-DL-cystine. Experimental conditions the same as in fig. 25.

tion. This was consistent with the findings after lower doses. The fall in radioactivity was not so pronounced as that which, in earlier experiments, occurred about three hours after the injection; there was still a substantial content after four hours.

Figure 30 illustrates the results following injection of S^{35}-L-methionine. The curve is in close agreement with that obtained after injection of S^{35}-DL-methionine.

To elucidate the rate at which another substance than an amino acid was excreted in the pancreatic juice under the same experimental conditions, the occurrence of radioactivity following injection of carrier-free $Na_2S^{35}O_4$ was studied. In a typical experiment (figure 31) the highest radioactive content was found only a few minutes after intravenous injection of 0.1 mC per kilogram of body weight. In this experiment samples were taken at one-minute intervals for the first five minutes. Radioactivity was

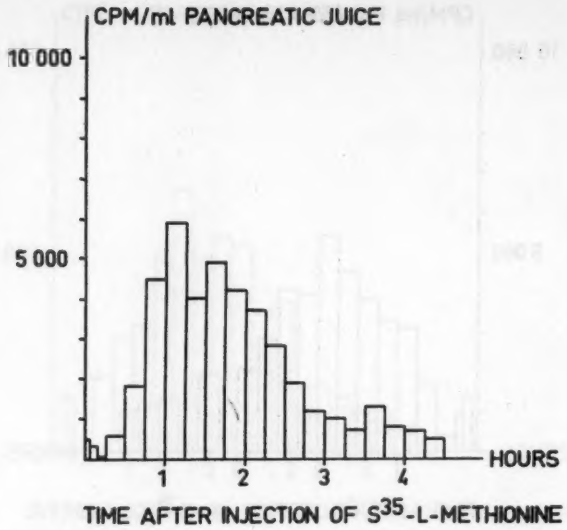

Fig. 30. Appearance of radioactivity in cat pancreatic juice after intravenous injection of S^{35}-L-methionine. Experimental conditions the same as in fig. 25.

already detectable after only one minute, and showed a maximum after three minutes. It then fell gradually and no peak was observed after 1–2 hours, as in the case of amino acids. In order to ensure measurable radioactivity in the pancreatic juice a dose of S^{35} was used which was ten times greater than that in the injection of labelled amino acids. The content of radioactivity in the juice was nevertheless much lower than that following injection of amino acids. The bulk of the observed radioactivity was TCA soluble (page 78).

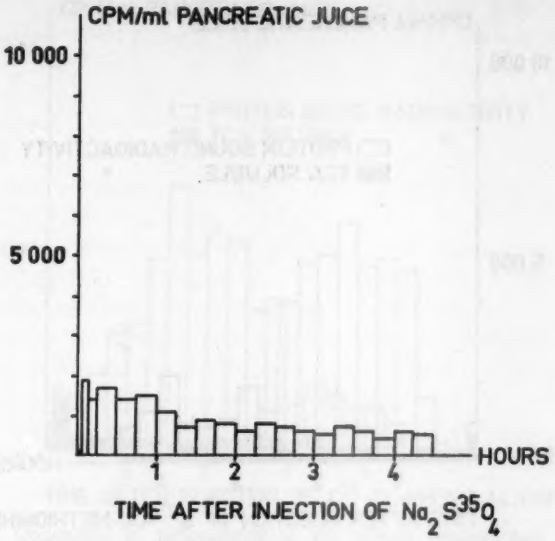

Fig. 31. Appearance of radioactivity in cat pancreatic juice after intravenous injection of $Na_2S^{35}O_4$. Experimental conditions the same as in fig. 25 with the exception that the pancreatic juice was collected every minute for the first 5 minutes after the injection.

DISTRIBUTION OF THE RADIOACTIVITY BETWEEN THE PROTEIN FRACTION AND THE TRICHLOROACETIC ACID SOLUBLE FRACTION

The occurrence of radioactivity in the protein fraction and the TCA soluble fraction was studied by precipitating the pancreatic juice proteins with TCA. Figure 32 illustrates the results of such separation after injection of S^{35}-DL-methionine. The radioactivity peak obtained during the first 15 minutes after the injection was in great part localized in the TCA soluble fraction. The radioactivity in the pancreatic juice 30 minutes after the injection and later was largely from S^{35}-methionine that had been incorporated into protein.

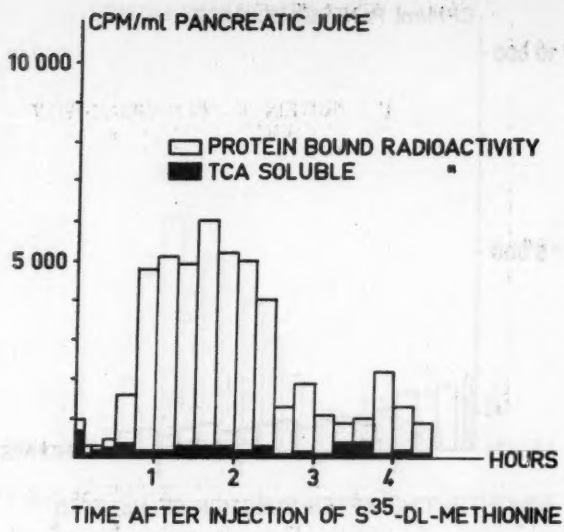

Fig. 32. Occurrence of radioactivity in the protein fraction and in the trichloroacetic acid (TCA) soluble fraction of cat pancreatic juice following intravenous injection of S^{35}-DL-methionine. Experimental conditions the same as in fig. 25.

Throughout the course of the experiment there was a small amount of radioactivity in the TCA soluble fraction; it remained fairly constant even though that in the protein fraction rose considerably.

Similar results were obtained on separation of a TCA soluble fraction and a protein fraction following injection of C^{14}-DL-phenylalanine (figure 33), S^{35}-L-methionine, and S^{35}-DL-cystine. At the peak found after about five minutes the radioactivity was TCA soluble. Thirty minutes and more after the injection the radioactivity was greatest in the protein fraction and low in the TCA soluble fraction.

By precipitating the proteins with TCA the occurrence of radioactivity in the protein fraction and the TCA soluble fraction of pancreatic juice was studied following injection of

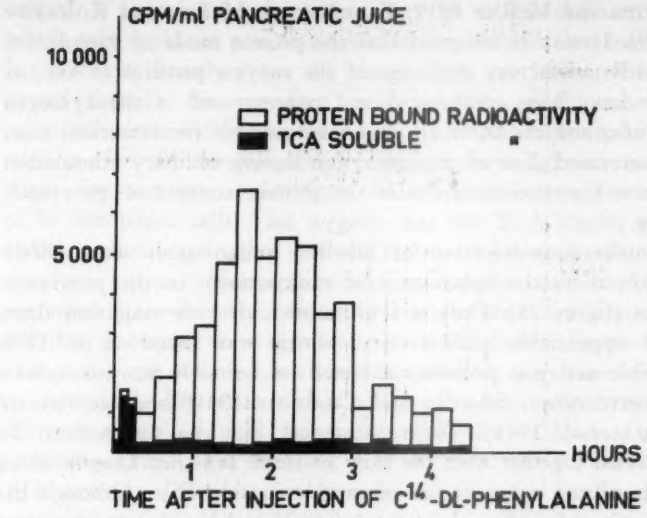

Fig. 33. Occurrence of radioactivity in the protein fraction and in the trichloroacetic acid soluble fraction of cat pancreatic juice following injection of C^{14}-DL-phenylalanine. Experimental conditions the same as in fig. 25.

$Na_2S^{35}O_4$. Virtually all radioactivity was present in the latter fraction; only very slight incorporation of radioactivity was found in the protein fraction.

DISCUSSION

By stimulating the pancreatic secretion with secretin and pancreozymin, a constant flow of fluid was obtained. The protein content was usually about 2–3 g per 100 ml at the start of stimulation but then fell to approximately 1 g per 100 ml after 30 minutes or so. During the course of the experiment the protein content declined and, as a rule, was somewhat below 1 g per 100 ml after 5–8 hours' stimulation (figure 24).—The probable explanation is that the resynthesis of proteins is insufficient to compensate for the heavy secretion induced by stimulation (KOMAROV, LANG-

STROTH and MCRAE 1939; LANGSTROTH, MCRAE and KOMAROV 1939). It may be assumed that the present mode of stimulation affords satisfactory discharge of the enzyme proteins as soon as they have been synthesized and concentrated in the zymogen granules and the latter are in a form suitable for excretion, since an increased dose of pancreozymin during ordinary stimulation does not conspicuously raise the protein content of pancreatic juice.

Intravenous injection of labelled amino acids was swiftly followed by the appearance of radioactivity in the pancreatic juice (figure 25). Only a few minutes after the injection there was appreciable radioactivity, which was found to be TCA soluble and was probably excreted in a similar way to sodium (MONTGOMERY, SHELINE and CHAIKOFF 1941) and bicarbonate (BALL *et al.* 1941). These substances may be assumed to be excreted together with the flow of fluid. It is not known which cells of the pancreas are permeable in this way. Although the secretion of enzymes and that of fluid and bicarbonate are regulated differently, the acinar cells are considered to be responsible for the entire secretion; this largely because no other cells have been found which might have the capacity to excrete products. GROSSMAN and IVY (1946), when studying the secretion of pancreatic juice in dogs, made observations suggesting that the centroacinar cells (*cf.* ZIMMERMANN 1927) took part in the excretion of pancreatic juice. The main purpose of those cells, it was thought, was to produce the fluid, and that of the acinar cells to produce enzymes. Although there is no conclusive evidence, the behavior in man that was described by LAGERLÖF (1939) tends to corroborate the hypothesis. Patients with pancreatitis had a normal fluid and bicarbonate secretion, but the secretion of enzymes was reduced.—It seems unlikely that inorganic material and fluid would be transported, to any major degree, through the layer surrounding the secretory ducts. These have a dense connective tissue coat, and the cubical epithelium which envelops the ducts probably does not possess secretory activity.

Little or no radioactivity is found in pancreatic juice 15–30 minutes after the injection, by which time the blood has a low radioactive content (HEVESY 1948; BORSOOK *et al.* 1950, and

others). It appears, therefore, that the TCA soluble radioactivity which does appear in pancreatic juice during the first few minutes has diffused directly from the blood; it is not likely to have passed through the excretory cells, for although the exocrine pancreatic tissue shows a high content of such radioactivity 15–30 minutes after the injection, there is only a very slight discharge, if any, into the juice at that time; the radioactivity is still retained by the acinar cells. This suggests that the TCA soluble products are discharged either intercellularly or by special cells.

In the present investigation the radioactive content of the pancreatic juice was high about 45 minutes after the injection, and then increased to a maximum after 1–2 hours (figure 26). This course was observed in all experiments that were conducted; no appreciable difference in the content of radioactivity between different amino acids, as reported by ROTSCHILD, HIRSCH and JUNQUEIRA (1957), was detected.

However, a difference between the radioactivity in the pancreatic juice proteins would be very probable after injection of various amino acids with the same specific activity. Two of the determining factors here would be the amino acid composition in the pancreatic juice proteins and its content in the acinar cells. If there is a high concentration of non-radioactive amino acids in the cells, isotope dilution will occur, thus reducing the specific activity in the formed proteins.

The present findings accordingly suggest that about 30–45 minutes elapse as from the incorporation into protein of amino acids taken up by the pancreatic cells until the proteins are ready for excretion. The synthesis of protein molecules takes only a minute or so (ZAMECNIK *et al.* 1956; LOFTFIELD 1957); the remaining time would thus be for transport in the cell and concentration of protein molecules in zymogen granules. The investigations also show that radioactivity appears in the pancreatic juice only a few minutes after intravenous injection of S^{35}-labelled sodium sulphate (figure 31). Similar results were reported by MONTGOMERY, SCHELINE and CHAIKOFF (1941) following injection of radioactive sodium. From these observations it may be assumed that the time required for transport of the enzyme proteins in the excretory ducts amounts to only a minute or so.

CHAPTER VIII

Incorporation of Intravenously Injected, Labelled Amino Acids into Pancreatic Juice Protein Fractions

The radioactivity occurring in pancreatic juice 30 minutes or more after intravenous injection of labelled amino acids was found to be localized mainly in the protein fraction (Chapter VII).

Only a few investigations designed to separate proteins of the pancreatic juice have been reported. Free electrophoresis was employed by MUNRO and THOMAS (1945), BYRNE *et al.* (1951) and GROSSBERG, KOMAROV and SHAY (1952); paper electrophoresis by DELCOURT and DELCOURT (1953) and ROTSCHILD and JUNQUEIRA (1956). KELLER, COHEN and NEURATH (1958) used chromatography. These investigations showed that the pancreatic juice contains a number of protein fractions. BYRNE *et al.* (1951), GROSSBERG, KOMAROV and SHAY (1952) and ROTSCHILD and JUNQUEIRA (1956) demonstrated that the various digestive enzymes were distributed among certain protein fractions.

In the present investigation paper electrophoresis was used for separating the protein fractions, and the distribution of some enzyme proteins among the fractions thus isolated was studied. The radioactive contents of the separated fractions were determined autoradiographically or by Geiger-Müller measurements on pieces cut from the paper electrophoretic strips.

RESULTS

PAPER ELECTROPHORETIC PATTERN OF PANCREATIC JUICE PROTEINS

The pancreatic juice used in this section was taken during the experiments described in Chapter VII. The secretion was stimu-

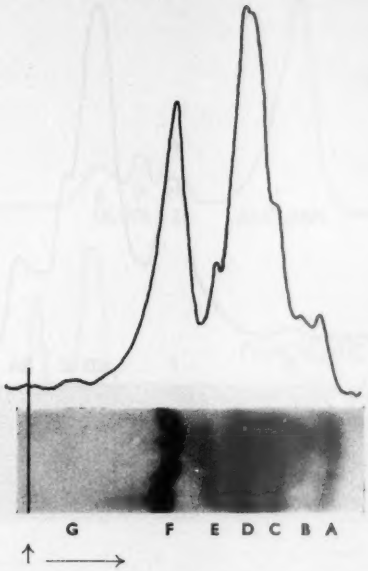

Fig. 34. Paper electrophoretic strip and corresponding photometric curve showing the separated protein fractions of cat pancreatic juice and their relative magnitudes. Seven fractions are separated and these fractions have been designated by the letters A to G. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

lated with secretin and pancreozymin. The protein content of the pancreatic juice was about 0.5–2 g per 100 ml.

Staining with amido black 10 B showed the presence of seven protein fractions in the pancreatic juice. These protein fractions have been designated by the letters *A* to *G*, as shown in figure 34. These symbols are employed to facilitate a description of the localization of the enzymatic activities in various protein fractions. Fraction *G* here is that which has the shortest migration; fraction *A*, that with the longest. Fractions *G* and *F* are distinctly separated; fractions *A* and *B*, and *D* and *E*, sometimes are not; and then only five fractions can be distinguished. The result will then be a paper electrophoretic strip as in figure 35. In deter-

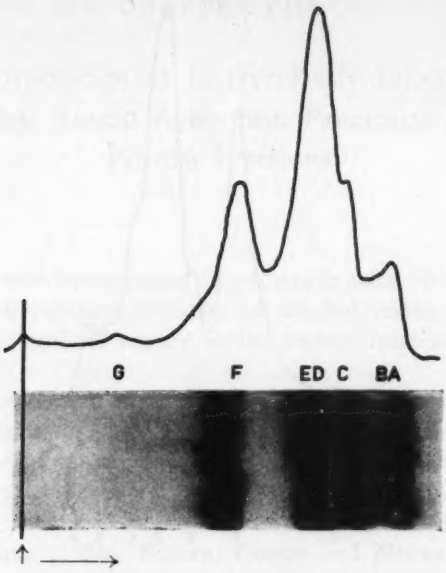

Fig. 35. Paper electrophoretic strip and corresponding photometric curve showing the separated protein fractions of cat pancreatic juice and their relative magnitudes. Five fractions are separated. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

mining the localization of the digestive enzymes, fractions *A* and *B*, and fractions *D* and *E*, were accordingly taken as single fractions as in figure 35. Electrophoretic strips of freeze-dried (page 30) and of natural pancreatic juice showed identical division into protein fractions.

Combined fraction *DE* was the most marked protein fraction, followed by *F*; fraction *G* was the least marked and, on some strips, difficult to detect; it was observed chiefly because it was the only one showing amylase activity (figure 38).

In figure 36 the protein fractions of pancreatic juice are compared with blood serum fractions from the same animals. The strips there were run coincidently in the same apparatus. Pan-

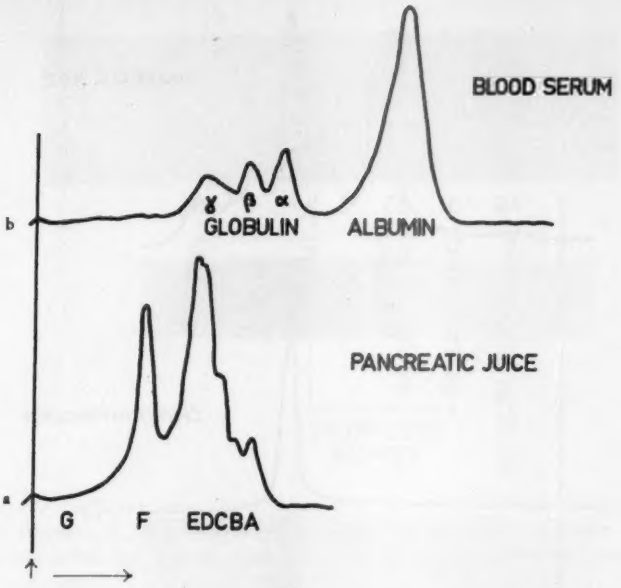

Fig. 36. Comparison between the paper electrophoretic patterns of cat pancreatic juice (a) and of cat blood serum (b). The paper electrophoresis was performed simultaneously in the same apparatus. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

creatic juice contained no fraction which corresponded to the albumin fraction of blood serum. The principal fractions corresponded to blood serum globulin. In figure 37 are compared the electrophoretic strips of a crystalline enzyme protein, bovine chymotrypsinogen (Worthington) and a corresponding one of pancreatic juice that was run simultaneously. It will be seen that protein fraction *DE* and the chymotrypsinogen had the same rate of migration.

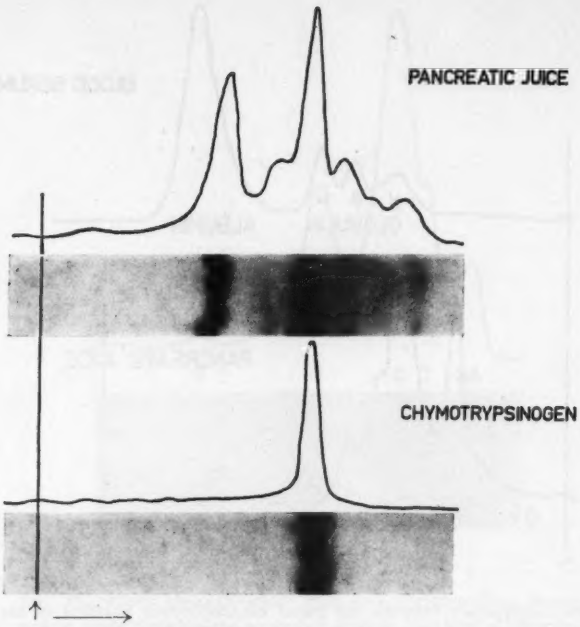

Fig. 37. Comparison between the paper electrophoretic strips and the corresponding photometric curves of cat pancreatic juice and crystalline bovine chymotrypsinogen. The electrophoresis was performed simultaneously in the same apparatus. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

DISTRIBUTION OF SOME DIGESTIVE ENZYMES AMONG PROTEIN FRACTIONS

Pieces of the electrophoretic strips were treated and the enzymatic activities determined as described on page 31. The distribution of the enzymes is shown in figure 38.

Fraction *G* was invariably found to contain amylase, the content of which was fairly uniform.

Lipase occurred mostly in fraction *DE*, but slight traces were observed in other fractions.

Proteolytic activity was present over the whole paper but

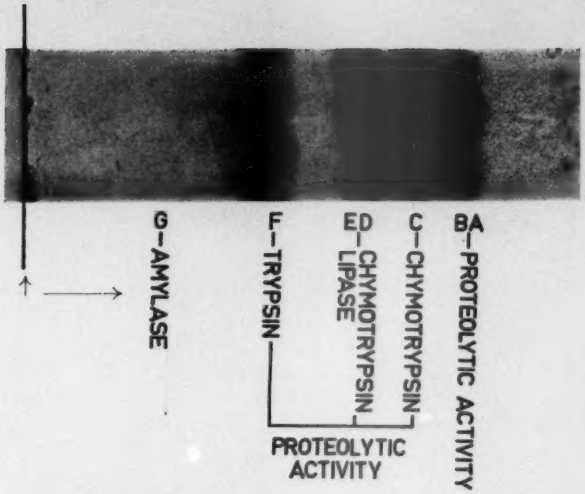

Fig. 38. The figure shows the distribution of enzymatic activities among protein fractions of cat pancreatic juice separated by paper electrophoresis. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

showed the highest content in fractions *AB*, *DE* and *F*. It was only detectable after activation with enterokinase.

Pronounced trypsin activity was found in fraction *F*. Trypsin activity was obtained only after activation with enterokinase.

The bulk of chymotrypsin activity was localized in fraction *DE*, but there was some in fraction *C*. Activation with trypsin was required for demonstrating chymotrypsin activity.

DISTRIBUTION OF THE RADIOACTIVITY AMONG PROTEIN FRACTIONS FOLLOWING INJECTION OF S^{35}-DL-METHIONINE AND C^{14}-DL-PHENYLALANINE

The localization of radioactivity in the various protein fractions was determined by autoradiographic recording of the dried electrophoretic strips or by Geiger-Müller measurements on the strips. For autoradiographic purposes a dose of 100 μC S^{35}-DL-

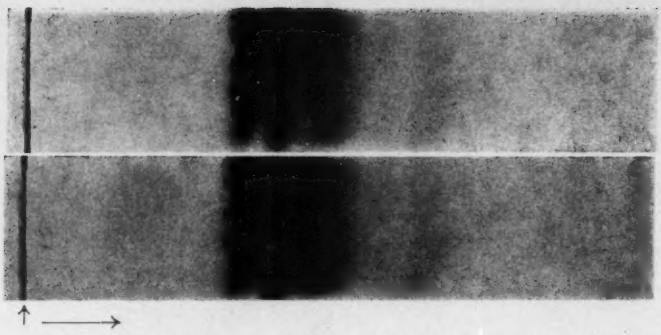

Fig. 39. Paper electrophoretic strip of cat pancreatic juice (a) and corresponding autoradiogram (b) (Industrex X-ray film, exposure time 3 months) from a cat injected with S^{35}-DL-methionine. Black areas correspond to high radioactivity. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

methionine per kilogram of body weight was injected in cats. During the first hour after the injection there was insufficient radioactivity in the pancreatic juice to darken the film; hence the autoradiograms taken were referable to times one hour or more thereafter. The exposure time ranged from three weeks to three months. Autoradiograms following injection of C^{14}-DL-phenylalanine were obtained after six months exposure, when a dose of 10 μC per kilogram of body weight had been injected in cats.

S^{35}-DL-methionine was incorporated into all of the protein fractions isolated (figure 39), though in the case of fraction *G* only slight radioactivity was found by Geiger-Müller measurement of punched out fragments of paper. The low activity therein may be attributable to the fact that fraction *G* constitutes only one per cent or so of the total protein of pancreatic juice. The radioactivity following injection of C^{14}-DL-phenylalanine showed exactly the same distribution pattern on the electrophoretic strips as that after methionine, but due to the low doses the autoradiograms were not so distinct. Instead, punched out pieces were examined in Geiger-Müller tubes (figure 40), thus enabling the radioactivity to be localized on the strips.

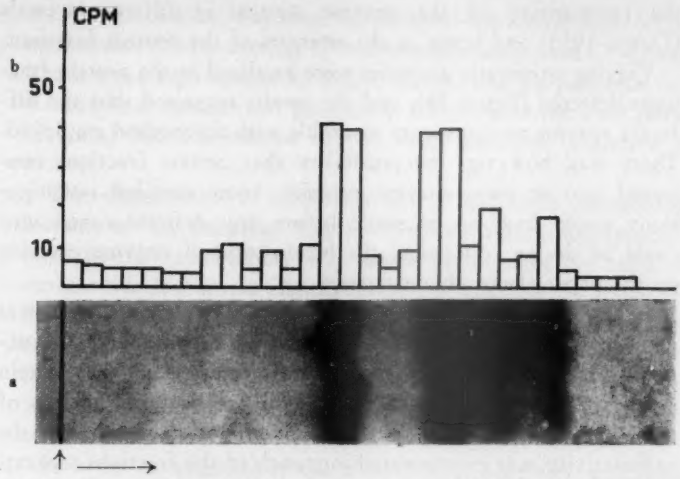

Fig. 40. Paper electrophoretic strip of cat pancreatic juice (a) and corresponding diagram (b) showing the distribution of radioactivity in the paper. The cat had received an intravenous injection of C^{14}-DL-phenylalanine. The vertical arrow indicates the starting line; the horizontal arrow, the direction of migration.

DISCUSSION

The distribution of radioactivity localized in the protein fraction was investigated by separating the pancreatic juice proteins by paper electrophoresis. With this method seven protein fractions were separated (figure 34), in which varying enzymatic activities were localized.—MUNRO and THOMAS (1945) using free electrophoresis, isolated five fractions in dogs; BYRNE *et al.* (1951) and GROSSBERG, KOMAROV and SHAY (1952), using another buffer solution, were able to separate six and ten fractions respectively. DELCOURT and DELCOURT (1953) and ROTSCHILD and JUNQUEIRA (1956) employed paper electrophoresis in dogs and rats, and isolated six and seven fractions respectively. In the present study no attempt was made to identify the protein fractions with those found in the above-mentioned investigations, since a different species of animal was used. Probably there are differences in

the composition of the enzyme content in different animals (DUKES 1955) and hence in the amounts of the protein fractions.

Varying enzymatic activities were localized in the protein fractions detected (figure 38), and the results suggested that the different enzyme proteins were separable with the method employed. There was, however, the possibility that certain fractions contained two or more enzyme proteins; more detailed investigations would have to be made before any definite conclusions could be drawn concerning the localization of enzyme proteins on the paper electrophoretic strips.

In this study the distribution of radioactivity on the strips was investigated after injection of S^{35}-DL-methionine and C^{14}-DL-phenylalanine. The radioactivity was localized in the protein fractions, and its magnitude was roughly proportional to that of the fractions (figures 39, 40). This investigation shows that the radioactivity was incorporated into each of the fractions separated by paper electrophoresis, and hence the results probably reflect the synthesis of all proteins in the pancreatic juice.

Summary and Conclusions

The aim of this investigation was to study the formation of pancreatic juice proteins, for which purpose labelled amino acids were used as precursors. The pancreatic uptake of those acids and their incorporation into proteins, as well as the excretion of the latter in the pancreatic juice were recorded by various methods.

The distribution of radioactivity in organs and tissues at varying intervals after injection of labelled amino acids was investigated by autoradiographic methods. The uptake by the pancreas was compared with that by other organs. Only five minutes after intravenous injection the concentration of radioactivity in the pancreas was several times greater than that in other organs. The maximum was reached in the pancreas about 30 minutes after the injection; the radioactivity then fell rapidly and, approximately six hours after the injection, was about the same as that in most of the other organs. No conspicuous differ-

ence in distribution was observed between the amino acids S^{35}-DL-methionine, S^{35}-DL-cystine, C^{14}-DL-phenylalanine, and C^{14}-glycine. There was a high uptake by organs which, like the pancreas, have a substantial synthesis of protein, as for instance the liver (serum albumin) and the mucosa of the small intestine (digestive enzymes).

The distribution of radioactivity in different morphologic sections of the pancreas was investigated autoradiographically. Most of the radioactivity was found in the exocrine cells; the content was low in the islands of Langerhans and the connective tissue. Histologic sections of pancreas at varying intervals showed that the radioactivity was localized diffusely in the exocrine cells for the first 30 minutes after injection, but from about one hour it was concentrated in the centers of the acini and in the excretory ducts.

In order to ascertain if the observed radioactivity was incorporated into protein or was in free amino acids, the protein-bound and non-protein-bound radioactivities were determined by precipitating and separating the pancreatic proteins with trichloroacetic acid. The radioactive contents of those fractions were recorded in normally fed, starved, and pilocarpine-treated mice and guinea pigs at different times after intravenous injection of S^{35}-DL-methionine. The non-protein-bound radioactivity reached its maximum about 10 minutes after the injection, irrespective of the secretory state of the pancreas. The maximum radioactivity per milligram of protein was found after 80 minutes in normally fed and in starved animals, but after approximately 20 to 40 minutes in those treated with pilocarpine.

These results show that the injected amino acids were swiftly incorporated into protein, and that excretion of newly produced, labelled proteins may occur about 40 minutes after the injection.

The various cell fractions of guinea pig pancreas—nuclear, zymogen granule, mitochondrial, microsomal and supernatant—were isolated by differential centrifugation in sucrose solution. Their proteins were precipitated and the radioactive contents thereof determined at intervals after intravenous injection of S^{35}-DL-methionine. For the first 40 minutes the specific activity was highest in the microsomal fraction but it then fell coincident

with a rise in the zymogen granule fraction. In the latter it was higher in pilocarpine-treated than in starved animals during the first 40 minutes, but then fell rapidly. The findings indicated that incorporation of radioactivity occurred later in the zymogen granule fraction than in others, and hence that the proteins in the former are synthesized by some other cell fraction, probably in the microsome fraction, and then accumulate in the zymogen granules.

The occurrence of radioactivity in pancreatic juice following intravenous injection of labelled amino acids was studied in cats whose secretion had been stimulated by intravenously injected pancreozymin and secretin. The flow of juice was continuous and the enzyme and protein contents were fairly constant. Already five minutes after the injection of labelled substances, radioactivity was detected in the pancreatic juice, though only in small amounts and largely in non-protein-bound form. Not until about 45–60 minutes had elapsed was any appreciable radioactivity found in the protein fraction, and a peak was recorded approximately 1–2 hours after the injection. The present findings accordingly suggest that about 30–45 minutes elapse as from the incorporation into protein of amino acids taken up by the pancreatic cells until the proteins are ready for excretion. The synthesis of protein molecules takes only a minute or so, the remaining time would thus be for transport in the cell and concentration of protein molecules in zymogen granules.

Pancreatic juice proteins were separated by means of paper electrophoresis. The distribution of digestive enzymes among the isolated protein fractions was investigated; the varying enzymes were localized in certain fractions. The radioactive contents of the isolated fractions were determined autoradiographically or by Geiger-Müller measurement of paper electrophoretic strips. The radioactivity was incorporated into each of the fractions separated by paper electrophoresis and the amount of radioactivity was roughly proportional to the magnitude of the protein fractions; hence the findings probably reflect the formation of all the different proteins in the pancreatic juice.

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